JOURNAL OF BACTERIOLOGY

VOLUME X

BALTIMORE, MD.
1925

Med.
Wm. T. Wilkins

CONTENTS

No. 1, January, 1925

A Streptothrix Isolated from the Blood in a Case of Acute Rheumatism with Remarks upon the Classification of Ray Fungi. T. Thjøtta and E. Gundersen 1
A Study of the Chemical Differentiation of Bacteria. Esther Wagner Stearn and Allen E. Stearn 13
Differential Staining of Living and Dead Bacterial Spores. S. A. Koser and J. H. Mills 25
Anaerobic Sporulating Thermophiles. Some Observations on a New Group of Bacteria. S. R. Damon and W. A. Feirer 37
On the Survival of *Bacterium typhosum* Intraperitoneally Implanted in Collodion Sacs. Frederick L. Gates 47
A New- or Delta-Type Streptococcus. Carrie Kirk Bryant 53
Old Stock Cultures as a Source of Bacteriophage. W. L. Mallmann 59
The Biochemistry of the Granulated Lactic Acid Bacteria from Cereals. E. B. Fred, W. H. Peterson and H. R. Stiles 63
Studies on Tellurium. The Value of Some Tellurium Compounds as Disinfectants. Lottie E. Munn with B. S. Hopkins 79
Biological Investigation of Peat. Arao Itano 87
The Occurrence of a Red Pigment Producing Organism in Corn Mash of the Acetone Butyl Alcohol Fermentation. E. B. Fred, W. H. Peterson and W. R. Carroll 97

No. 2, March, 1925

Indol and Skatol Determination in Bacterial Cultures. Carl R. Fellers and Ray W. Clough 105
Concerning the Physiology of *Thiobacillus thiooxidans*, an Autotrophic Bacterium Oxidizing Sulfur Under Acid Conditions. Robert L. Starkey. 135
Concerning the Carbon and Nitrogen Nutrition of *Thiobacillus thiooxidans*, an Autotrophic Bacterium Oxidizing Sulfur under Acid Conditions. Robert L. Starkey 165

No. 3, May, 1925

Contributions to the Knowledge of *Vibrio cholerae*. I. Fermentation of Carbohydrates and Polyatomic Alcohols by *Vibrio cholerae*. Keizo Nobechi 197
The Reducing Properties of Microorganisms with Special Reference to Selenium Compounds. Victor E. Levine 217
Studies on Certain Characteristics of *Clostridium chauvei* and *Clostridium edematis*. Joseph P. Scott 265

No. 4, JULY, 1925

Recent Work on the Descriptive Chart and the Manual of Methods. H. J. Conn, K. N. Atkins, J. H. Brown, B. Cohen, G. J. Hucker and F. W. Tanner 315
Studies on the Metabolism of *B. botulinus* in Various Media. XXVI. E. Wagner and K. F. Meyer in cooperation with C. C. Dozier 321

No. 5, SEPTEMBER, 1925

Clostridium multifermentans in Chocolate Cream Candies. Grace A. Hill .. 413
Relation of Temperature to the Growth of Thermophilic Bacteria. Fred W. Tanner and George I. Wallace 421
Hydrogen Sulphide Production by Anaerobic Spore-Bearing Bacteria. Morton Charles Kahn 439
Further Cultural Characteristics Differentiating *B. chauvoei* from Other Anaerobes. A. S. Schlingman and A. W. Haines 449
Indol Studies. Walter L. Kulp 459
On Bacterial Variation. J. Ørskov and Arnd Larsen 473
Studies in Microbic Heredity. I. Observations on a Primitive Form of Sexuality. (Zygospore Formation) in the Colon-Typhoid Group. Ralph R. Mellon 481

No. 6, NOVEMBER, 1925

A Study of Anaerobic Bacteria. J. Howard Brown 513
The Bacteriological Content of Market Meats. C. M. Brewer 543
Long Tube Method of Cultivating Microorganisms with Observations on Mobile Colonies in Liquid Medium. Lewis B. Bibb 561
Club-Formation by *Actinomyces hominis* in Glucose Broth with a Note on *B. actinomycetum comitans*. Stanhope Bayne-Jones 569
Studies in Microbic Heredity. II. The Sexual Cycle of *B. coli* in Relation to the Origin of Variants. With Special Reference to Neisser and Massini's *B. coli-mutabile*. Ralph R. Mellon 579

A STREPTOTHRIX ISOLATED FROM THE BLOOD IN A CASE OF ACUTE RHEUMATISM WITH REMARKS UPON THE CLASSIFICATION OF RAY FUNGI

T. THJØTTA AND E. GUNDERSEN

From the Bacteriological Laboratory of the Norwegian Army, Christiania

Received for publication June 1, 1924

In the beginning of August, 1923, a medical student was admitted to the Military Hospital of Christiania from a training camp, suffering from acute rheumatism. During a five months stay in the hospital the patient passed through a severe attack of rheumatism, starting with a sore throat, then having effusions of the joints, pleuritis, pericarditis and bronchitis. Except for a short period in which he was free from fever in September, the patient suffered from a fever of moderate intensity from the beginning of the disease to the recovery.

We will not go into details as to the clinical picture and the course of the disease, but exclusively deal with the bacteriological findings. Three blood cultures were made from the patient, one during each of the two fever periods, and one after recovery. Furthermore, two sputum examinations and one complement fixation test were carried out.

The blood-cultures were made in veal broth, to which had been added 0.2 per cent of secondary sodium phosphate, instead of sodium chloride. The pH was 7.8. Sterilization of the medium was carried out at 115°C. for one-half hour. To 100 cc. of this broth 10 cc. of the blood were added and the culture so prepared incubated at 37°C.

In both of the first two cultures we obtained growth of the same microbe, while the culture taken after recovery proved sterile. The two positive culture tests were made by two different examiners, at different times one and one-half months apart

in different hospitals (the patient being moved to a civil hospital where the second culture was taken). Two different batches of culture media were used.

The isolated microbe showed the following characters:

The cultures gave no diffuse turbidity, and no growth could be observed before the lapse of two weeks, when small woolly colonies began to form in the flask on the layer of blood covering

FIG. 1

the bottom. A few days later more small colonies could be seen extending from the bottom along the wall of the flask (fig. 1). A distinct radial structure of the colonies was observed as they grew larger. From some of the uppermost colonies which had reached the surface of the medium a white, scaly pellicle was formed, which grew gradually out over the surface. The pellicle (fig. 2) was of a fairly dry, scaly appearance while

the bottom colonies mentioned were woolly, round and from 2 to 3 mm. in diameter. The medium between the bottom- and the surface-growth remained perfectly clear. A distinct odor of mold was observed from the culture.

On microscopic examination of the colonies in the bottom, we found a number of interwoven, branching, homogeneous,

FIG. 2

Gram-positive filaments without segment formation, without clubs and without spores (fig. 3). Where the filaments were well separated, the distinct true branching was well pronounced (fig. 4). This uniform picture stands out in sharp contrast to the microscopic picture seen in films from the surface growth (fig. 5), where branching filaments are seen side by side with

thick, twisted filaments and threads that show a distinct breaking up into several ovoid, strongly refractive bodies.

When grown on solid media the organism develops rapidly. On sugar agar (lactose) it forms after from one to two days'

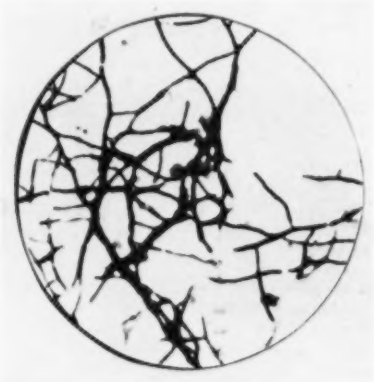


FIG. 3

FIG. 4

FIG. 5

growth fairly large, compact colonies with a smooth and shiny surface and a well defined circular edge. After a few days more these colonies are surrounded by a collar of white powder-

like material which gradually creeps up on the colonies and at last covers them more or less completely (see fig. 6, where some colonies still show the shiny surface and the collar, while others are covered by the white powder). If grown on a poor medium, such as a plain water agar, the organism develops much more slowly, and the colonies are smaller and drier than on the more favorable media. On the water agar the colonies are covered by the white powder from the beginning and consequently always show a dry white picture. During their further growth on water agar the colonies constantly increase in size and form concentric rings or radial colonies (fig. 7).

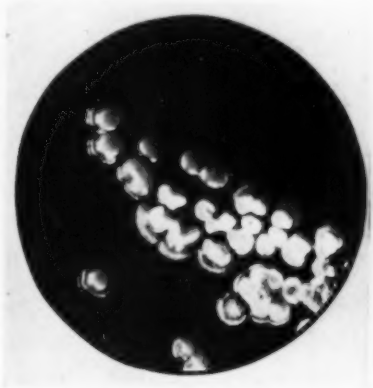


FIG. 6

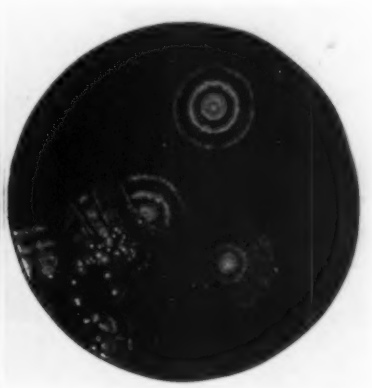


FIG. 7

When a colony on water agar was cut through and examined from the side it was found to consist of two distinct parts, one growing downwards into the medium, the other upwards. The latter carried on its surface several elements which extended above the medium (fig. 8).

On microscopic examination the growth on the surface of solid media showed a picture completely different from the one seen in the bottom colonies in the fluid medium. The surface colonies consisted of morphologically different elements. The most prominent were Gram-positive coccoid forms, single, in pairs like diplococci, or situated two in each end of a short

Gram-negative rod (giving the picture of a diphtheroid rod). Here and there Gram-negative threads were seen, that showed in their protoplasm more or less Gram-positive elements of the kind described. These elements seemed to develop as enclosures in the thread, and it could be seen in several films how the round, Gram-positive elements became free from the threads. Now and then completely Gram-negative threads were seen, obviously the remnants of threads that already had yielded their tribute in the production of the free Gram-positive bodies.

It must be assumed that these bodies are the spores formed by the organism. The spore formation takes place only in the

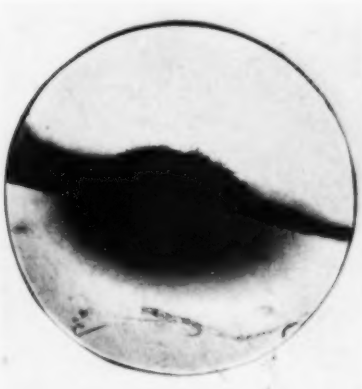


FIG. 8

FIG. 9

surface colonies and most abundantly in the colonies on the poor water agar medium. On this medium the spore formation could be seen macroscopically as the structure of the white powder covering the colonies and the aerial mycelium is, as shown in figure 8, distinctly visible under a low power lens. On the surface of the richer medium (lactose agar) the spore formation does not appear as early as in the poorer medium, but when it does appear it is typical. On the surface of the fluid medium the spore formation was also seen, but not so distinctly, nor has the surface on the fluid medium the decided powdery appearance of that on the solid medium. In the bottom colonies, however, we never find any spore formation.

On cultivating this organism in ordinary broth, it showed a distinct growth optimum. On measuring the diameter of the bottom colonies in a series of tubes with a pH varying from 8.2 to 5.0 we always found the most luxuriant and most rapid growth in the tubes having a pH of 7.4.

On the surface of ox serum the organism grew typically without any peptonizing of the medium, while in nutrient gelatin it showed a quick and complete liquefaction.

When tested on animals, it proved to be nonpathogenic; large amounts of cultures were injected intravenously, intraperitoneally and intratracheally into rabbits and guinea-pigs, without any apparent effect.

The organism isolated from the blood of this patient shows the following properties:

It develops in the depth of nutrient broth with the formation of Gram-positive, true branching filaments which are free from segments and spores. On the surface of solid and fluid media, it forms a well developed aerial mycelium which undergoes distinct spore formation. It liquefies gelatin, but does not peptonize serum. It is not pathogenic for rabbits and guinea-pigs.

According to its morphological and cultural properties the organism in question belongs to the large group called by Petruschky (1913) Thread-fungi or Trichomycetes. These fungi produce a fine mycelium with true branches, and develop spores to a somewhat varying degree. In the overwhelming literature on these fungi we find them described under many different names. Even the same name is used by different authors to signify different organisms. This existing chaos is accentuated by the fact that many authors often depend largely or entirely on a morphological and a clinical description. Organisms of this type are found under the names of Actinomyces, Discomyces, Streptothrix, Nocardia, Oospora, etc.

A completely satisfactory classification of these fungi has not yet been affected. The simplest and most appropriate was up to the last few years that of Petruschky, who divided the microbes in question into the subgroups Actinomyces and Streptothrix. The main difference between these subgroups is,

according to Petruschky, the formation of clubs in the animal host by the Actinomyces. This point has, however, been questioned by several authors, and as this difference applies only to the fungi found in lesions in man or animals, it cannot be used in any classification where the microbe in question is found outside the animal organism and proves to be without pathogenic properties.

A classification of these fungi should, therefore, like the classification of all bacteria be based upon simple morphological and cultural properties that may be reproduced where and whenever desired. These properties should be stated very carefully by the bacteriologist in describing a given type or species. To gain more knowledge about these fungi the description should also in so far as possible keep to the main lines of a scheme that has proved adequate. Such a scheme was, to our knowledge, first given by Ørskov (1923), who published his results based on an extensive study of these organisms as late as 1923.

Working with a number of fungi ("ray fungi") partly isolated by himself and partly by other authors known under scientific names, Ørskov studied the morphological and cultural development of the fungi, using the method developed by himself for one-cell culture (1922). Without destroying the organism by fixing and staining, Ørskov was able to follow the development of one single spore or mycelial thread, through different stages and into the spore stage. He could determine a distinct type of growth for the different fungi, and was able to assign simple and easily discovered properties that might allow of a classification. Ørskov finds that all the fungi examined by him may be classed in three groups: I, II, and III. When he applied names to these groups he made use of the old names that are most in accordance with the characters of his fungi. He calls the different groups Cohnistreptothrix, Actinomyces and Micromonospora.

Group I Ørskov names Cohnistreptothrix, to indicate that the fungi belonging to this group morphologically and culturally behave like the organism described by Cohn in 1873 and not like that described under the same name by Corda in 1839,

a fungus belonging to the molds. In group I Ørskov places microbes known under the names of *Actinomyces hominis* Landsteiner, *Actinomyces madurae* Vincent, *Nocardia Dassonvillei*, *Actinomyces* Bostrøm, *Actinomyces albus* Berestnew, and a number of organisms simply named Streptothrix.

All of the fungi of this group are homogeneous. They form spores that grow out to a unicellular mycelium, and the filaments do not divide into segments. From this so-called "substratum" mycelium and aerial mycelium grows out, and this divides after some time into regular divisions of threads. The formation of the aerial mycelium is always seen earlier and to a greater extent on poor than on rich media. The colonies on a solid medium are firm, and cannot be removed from the medium without the use of an instrument which removes the whole colony, along with a piece of the agar. In fluid media these fungi grow on the bottom as woolly colonies and with the formation of a surface pellicle, when some of the bottom colonies reach the surface. The spore formation is seen macroscopically as a white powder that covers the growth. As a rule the color is white, but it may differ even in the same strain, and therefore cannot be used as a means of differentiation. The spore-bearing aerial mycelium is thicker than the substratum mycelium and often shows twisted filaments. The spores are more resistant than the filaments.

The fungi belonging to group II are less uniform than those of group I. This is especially the case as regards the cultural characters; the morphological properties show a greater uniformity. Group II is subdivided into A and B. Of these subgroups A forms an aerial mycelium, while this is not the case with B. In all other respects the two subgroups are alike.

The members of group II A develop a substratum mycelium that is undivided in the beginning of the growth, but becomes divided into segments later on. The aerial mycelium is developed very early and can only be distinguished with the greatest difficulty from the substratum mycelium; it is as a rule so small that it cannot be seen with the unaided eye. The spores are not more resistant than the mycelium filaments. On solid

media the colonies may be fixed to the medium or rest quite loosely on the surface. In fluid media the fungi of this class grow only very sparingly at the bottom, while they quickly form a pellicle on the surface.

In group II A we find fungi of the following names: *Actinomyces corneae* Løwenstein, *Streptothrix* Deycke, *Actinomyces* Eppinger, *Nocardia* Eppinger, *Cladothrix* Eppinger, *Streptothrix ruba.*

The members of group II B are distinguished from those of II A in that they do not form an aerial mycelium. Some of these fungi have a tendency to develop into a so-called angular growth, like that described by Nocard for *Nocardia farcinica* and by several authors for the diphtheria bacillus. In this division Ørskov has grouped the following organisms: *Streptothrix polychromogenes* Vallée, *Nocardia farcinica, Streptothrix canis.* To this group he also assigns the sole anaerobic organism that he has studied, a fungus grown from a case of actinomycosis in man. With the exception of its anaerobic property this organism was in all respects like the others of this group.

In group III Ørskov places only one ray fungus, namely, *Streptothrix chalceae,* which develops a fine mycelium. In the extremely fine branches of the mycelium small oval spores are formed. This organism does not form a pellicle on the surface of liquid media.

It is evident from the classification of Ørskov that fungi that have called forth the clinical disease of actinomycosis or of streptothricosis are to be found in both group I and group II. This fact is in full harmony with the former history of these fungi. The authors have discussed the question as to whether the "real" actinomycosis fungus was an aerobic or an anaerobic organism. According to Ørskov several organisms apparently may call forth the clinical pictures of actinomycosis or streptothricosis. It seems then incorrect to describe the organism found in actinomycotic lesions as an "actinomyces" without subjecting it to a rigid bacteriological examination. It will undoubtedly be of more value to bacteriological science to study the microbe in question very closely first, then put it into a scheme of classification and at last conclude that this microbe, which belongs to

such and such a group has in the case in hand been a producer of actinomycosis. This is the routine that Ørskov has indicated, and we have in the work at hand tried out his mode of classification in actual practice.

It must be clear that our microbe very naturally and without any doubt falls into group I of Ørskov. It has in fact been very much easier to identify our microbe by the characters given by Ørskov than by any description found elsewhere in bacteriological textbooks, etc. We will not, any more than Ørskov himself, claim that this classification is ideal. Ørskov is well aware of the fact that probably more groups will have to be included. Probably a group will have to be made for anaerobes, as undoubtedly some of these fungi only grow in the absence of oxygen.

We feel justified in claiming that the microbe isolated by us from the blood of a patient suffering from a severe attack of rheumatism is a true fungus, and that it falls into group I of Ørskov and may be given the well known name of "Streptothrix" or according to Ørskov "Cohnistreptothrix." We will now have to answer the question as to its pathogenicity. It is obvious that we cannot point out any symptoms in the patient that naturally would indicate infection by a fungus. The clinical picture was without any doubt that of acute rheumatism. We therefore feel that our microbe cannot be taken as the etiologic agent in this case. We have tried out the complement fixing property of the patient's blood after his recovery, but were not able to detect any such property against the organism isolated from the blood. When we further take into consideration the absolute lack of pathogenicity in animals, we assume that our microbe has been a mere saprophyte without any relationship to the disease.

How has this microbe gained entrance into the blood of the patient? We have only one explanation to offer. It was probably present in the respiratory tract of the patient as a saprophytic organism at the time when the illness began. It has been shown by Ørskov that organisms of this kind are not uncommon in soldiers, where he found them in the throat and on the tonsils.

If our patient was a carrier of an organism like the one isolated it may have broken through the barriers of the body and passed over into the circulating blood during the severe sickness of the patient, when the defence of the body was low. The microbe, however, was not pathogenic to the patient and consequently did not call forth any pathological lesions; nor did it cause the production of antibodies.

Our streptothrix strain is then an example of a non-pathogenic streptothrix. In the literature there are a number of communications dealing with strains quite like ours (i.e., morphologically and culturally) that have caused severe disease with a fatal result. As a rule the origin of the disease caused by these organisms seems to be the respiratory tract, and the first clinical sign of disease a bronchopneumonia. Thus Løhlein (1909) describes a fatal case of that nature, where the fungus, as far as we can see, was quite like ours in all respects except pathogenicity in guinea-pigs.

A number of cases of this nature are known and we do not wish to go into detail on this point. However, we would urge that in every case where an organism belonging to the species mentioned here is found as the cause of disease the description of the case should not only be a clinical or pathological one, but a thorough study should be made of the organism, and an endeavor made to place it in a system which is built upon distinct biological characters, as for instance that of Ørskov.

REFERENCES

LØHLEIN, M. 1909 Zeitschr. f. Hyg., **63**, 1.

ØRSKOV, J. 1922 Jour. Bact., **7**, 507.

ØRSKOV, J. 1923 Investigation into the morphology of the ray fungi. Communications de l'Institut Serotherapique de l'Etat Danois, **14**.

PETRUSCHKY 1913 Kolle u. Wassermann, Handbuch der path. Mikroorganismen, **5**, 267.

A STUDY OF THE CHEMICAL DIFFERENTIATION OF BACTERIA

ESTHER WAGNER STEARN AND ALLEN E. STEARN

From the Public Health Laboratory and the Division of Physical Chemistry, University of Missouri, Columbia, Missouri

Received for publication June 1, 1924

The study of bactericides and bacteriostats and the study of staining reactions has brought about an investigation involving a differentiation of bacteria. This differentiation is based upon the resemblance of a bacterium to an organized chemical unit in its response to reagents.

It has been shown (Stearn and Stearn, 1924) that bacteria react at varying values of pH to dyes in the same way as do proteins. At low pH values they tend to combine with acid dyes, at higher pH values with basic dyes. In this manner their amphoteric character is established. The Gram method for classifying bacteria seems to rely for its specificity upon the fact that bacteria act as charged particles, some more highly charged than others. It is thus a truly chemical differentiation. The determination of the nature of the response of a bacterial unit to a chemical reagent, as well as the constancy of such a response, is important.

Just as chemical substances are known to decompose, forming new compounds of definite, though different, chemical nature and characteristics, so also it seems that bacteria will mutate as to morphology, physiological activity, chemical constitution and pathogenicity as their environment is altered. Loehnis and Smith (1916) claim that a single species may pass through as many as twelve to fourteen distinct morphological forms, varying from tiny filterable "gonidia" to large unorganized masses of "symplasm" formed by the fusion of smaller elements. Revis (1913a) has pointed out the effect of dyes such as malachite

green and brilliant green on the mutation of bacteria. From an original typical culture of *Bact. coli* two strains arose showing permanent changes in physiological activity as tested on milk, lactose, glucose, etc., but not in growth. Increasing amounts of malachite green changed a typical *Bact. coli* to an organism consisting principally of long filaments which were neither physiologically nor culturally colon bacilli. Many other instances may be cited (Smirnow, 1916; Clark, 1919; Scales, 1917; Dawson, 1919; Kellerman and Scales, 1917; Revis, 1913b) in which environment changed the morphological and biological characteristics of bacteria. Instances in which the Gram character was altered (Nikitin, 1908; Neide, 1904; Kantorowicz, 1909; Eisenberg, 1910; Sheppe and Constable, 1923) and also in which the chemical constitution of an organism assumed variations (Dawson, 1919), may be cited.

It seems that the prime difference between an ordinary chemical molecule and a bacterial unit may be in the susceptibility to environmental conditions. Gradual acclimatization must take place where organic life is concerned if life is to be preserved.

The authors wish merely to present experimental data bearing on the following two points: (1) Bacteria act as organized charged units. At ordinary values of pH they are negatively charged to a greater or lesser degree, as demonstrated and measured by their varying affinity for basic dyes. Measurements of this affinity, for any one species of bacteria at any one pH value, can be duplicated. This means that they exhibit, under ordinary conditions, acidic properties. (2) Bacteria can be oxidized, increasing their inherent acidity, just as is the case with any organic compound. The degree of oxidation depends on the relative power of the oxidizing agent employed, as shown by the relative effect of iodine and potassium dichromate.

EXPERIMENTAL

The following organisms were used for these experiments:

A. Gram-positive

1. *Bacillus cereus*
2. *Bacillus subtilis*

3. *Micrococcus tetragenus*
4. Streptococcus (non-hemolytic)
5. *Streptococcus pyogenes*
6. *Corynebacterium diphtheriae*

B. Gram-negative

1. *Bacterium coli*, var. *communior*
2. *Bacterium aerogenes*
3. *Bacterium typhosum*
4. *Bacterium dysenteriae* (Shiga)

All cultures were obtained from the American Museum of Natural History, except that of *C. diphtheriae*, which was obtained from the Public Health Laboratory, Columbia, Missouri.

Thin smears of the organisms were made on glass slides, air dried, and then slightly warmed. After staining with carbol

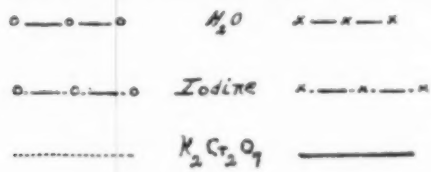


FIG. 1. KEY TO FIGURES 2–5 (ON SUCCEEDING PAGES)

basic gentian violet for two minutes, they were flooded with one of the mordants listed for three minutes. They were then decolorized with acetone and examined at once, comparing the slides repeatedly. At each pH studied one smear was run with each of the following mordants: (1) distilled water at varying values of pH, (2) Lugol's iodine at corresponding values of pH, and (3) 1 per cent $K_2Cr_2O_7$ at corresponding values of pH. These pH values were adjusted in acid solutions by means of acetic acid-acetate mixtures, and in the more alkaline solutions with ammonium hydroxide-ammonium chloride.

In the same way a second set of smears was run using carbol acid fuchsin as stain.

In our curves we have divided the above bacteria into three groups, placing *C. diphtheriae* and *Bact. dysenteriae* in a group by themselves. The former, while classified as Gram-positive,

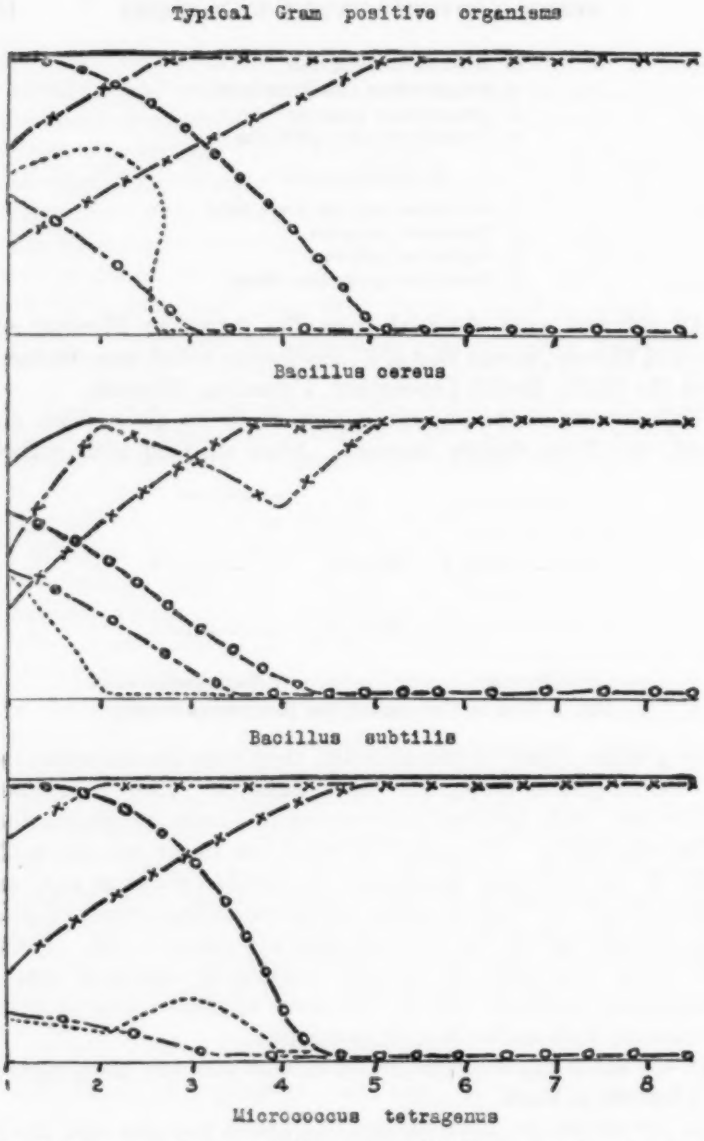


FIG. 2. ABSCISSAE REPRESENT VALUES OF pH, ORDINATES ARE ARBITRARY FUNCTIONS OF THE INTENSITY OF RETAINED COLOR

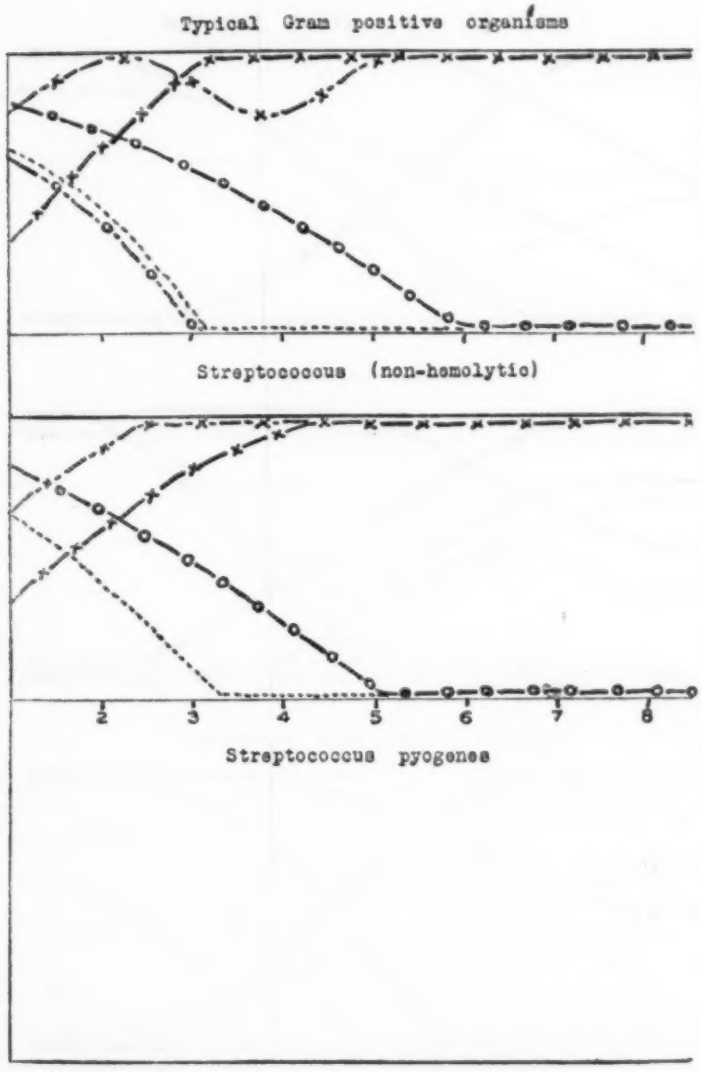


FIG. 3. ABSCISSAE REPRESENT VALUES OF pH, ORDINATES ARE ARBITRARY FUNCTIONS OF THE INTENSITY OF RETAINED COLOR

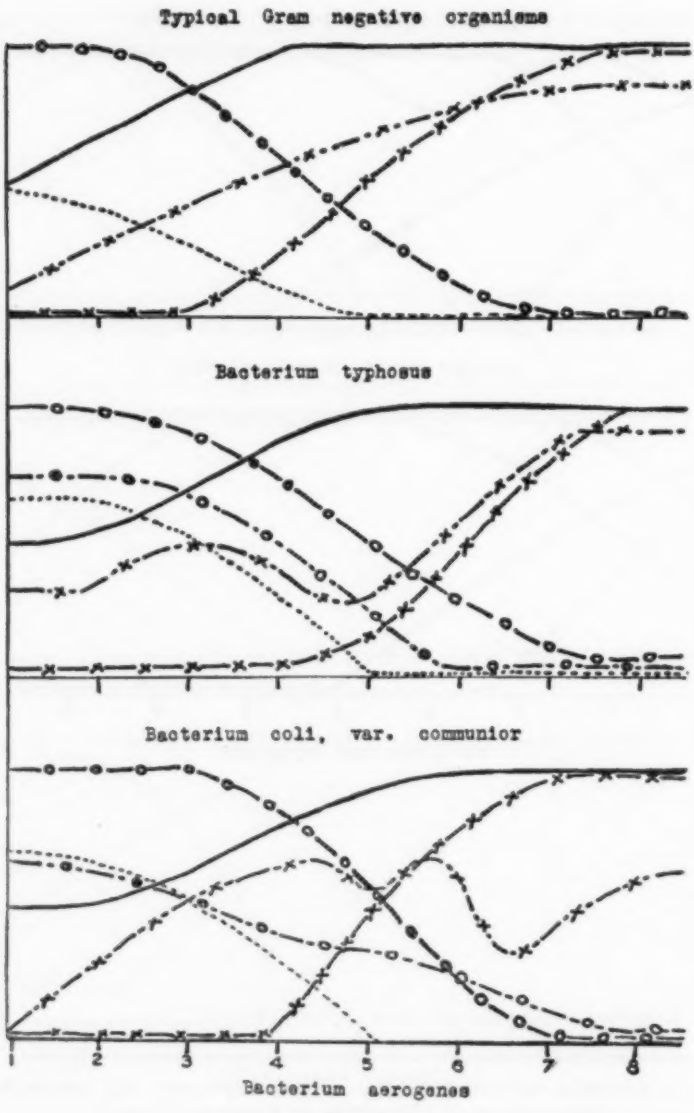
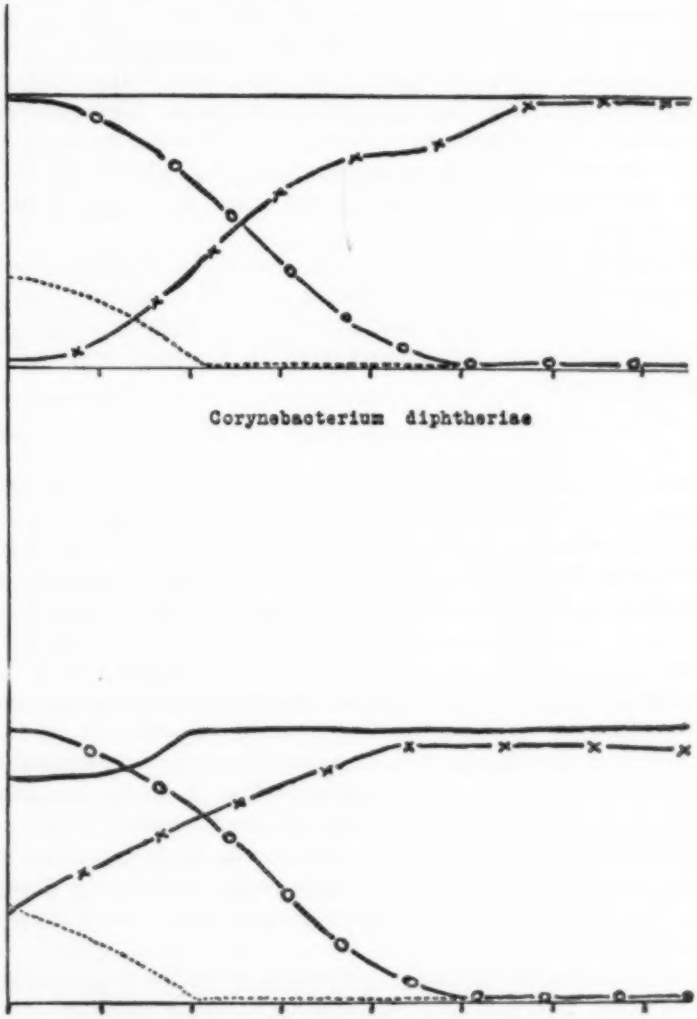

Typical Gram negative organisms
Bacterium typhosus
Bacterium coli, var. communior
1
2
3
4
5
6
7
8
Bacterium aerogenes

FIG. 4

Variable organisms



Corynebacterium diphtheriae

Bacterium dysenteriae (Shiga)

FIG. 5

is known to be but weakly so, even slight changes in certain environmental conditions, such as pH, rendering it negative. *Bact. dysenteriae*, while it is consistently Gram-negative, was shown by Kligler (1918) to be distinctly more susceptible to the bacteriostatic action of basic dyes than other Gram-negative organisms, and thus to approach in character Gram-positive organisms. A comparison of our curves shows a pronounced similarity between these two organisms, and a behavior on their part intermediate between that of the other two groups in reaction to dyes.

It may be pointed out that our results on *Bacillus cereus*, yielding an isoelectric range centered at a pH of about 3, are in good agreement with the results of Winslow, Falk and Caulfield (1923) who found an isopotential point for this same organism at a pH of about 3.

DISCUSSION

A most fundamental question in bacteriology is what shall constitute a reliable basis of classification. This question seems as yet unsolved. Obviously classification on the basis of morphology alone may have little significance, and might be almost as dangerous as classiying chemicals by their crystal form and size. The usual classification based upon the morphology of bacteria cannot be regarded, according to Hiss and Zinsser (1916), as "anything more than a convenient make-shift pending the day when the finer structure and true biological relations of the various bacteria shall have been more accurately investigated." To push the chemical analogy a little farther, it would be unwise to conclude that different morphology need necessarily denote essentially different nature. For example we know two forms of phosphorus entirely different in appearance (morphology), and differing widely also in that the one form is very toxic while the other is not.

At present bacteriologists are relying more on the constancy of character as shown by cultural studies, staining reactions, and pathogenic properties. "Reactions with specific immune sera . . . . seem to indicate a fundamental chemical difference

in the constitution of bacteria often morphologically very much alike" (1916). It would seem that this fundamental chemical difference in the constitution of bacteria is in some way or other rapidly or slowly altered by environment in a manner somewhat analogous to that in which chemical compounds are changed. This is brought out by the data presented in the preceding section.

A study of the graphs will reveal that:

1. Certain bacteria are easily oxidizable: *Bacillus cereus*, etc.
2. Other bacteria are less easily oxidizable: *Bacterium coli*, etc.
3. Any bacterial cell responds to degrees of oxidation, i.e., oxidizing agents of different oxidizing power will change its properties in the same direction but to varying degees.

While it is not brought out in the graphs, the point was repeatedly confirmed that treatment of an oxidized smear with a reducing agent such as stannous chloride tended to restore the smear to its original state, so far as staining reaction was concerned, thus counteracting the effect of the previous oxidation.

These graphs further reveal that:

1. Gram-positive organisms have a limited isoelectric range which is centered at a comparatively low pH, around 2 to 3.
2. Gram-negative organisms have a wider isoelectric range at a higher pH, i.e., centered about a pH of 5.
3. Certain organisms which we have termed variable have an isoelectric range centered somewhere between these two extremes.

The above comparison means that organisms vary in the magnitude of their inherent negative charge at any one pH value. One group of organisms shows a comparatively great affinity for basic dye through a wide pH range, while another group exhibits such an affinity to a much lesser extent. In all cases the affinity for basic dye increases with increasing pH as well as with an increased state of oxidation of the organism.

Oxidizing agents are not the only chemical reagents which will alter the properties usually characteristic of a strain. The constituents of media will also "react" on bacteria, changing their characteristics. Dawson (1919) has shown that "by changing the character of the media upon which a given variety of bacteria

is grown the chemical constitution of the bacteria can be made to vary, and, with it, certain *biological characters* such as agglutinability with immune serum, and the formation of sugar splitting enzymes. In some instances the variations practically amount to the production of a new strain. Morphological changes appear to be relatively unimportant."

Rosenow (1914), in his very interesting paper on "The transmutations within the streptococcus-pneumococcus group," points out that "the changes observed are not due to mixtures nor to so-called "mass-selection," but to actual changes wrought under the influence of changed environment." He also points out that, as the morphology and the cultural and agglutinating properties of the two strains of pneumococcus change, the pathogenicity changes likewise. Thus, since sudden changes in environmental conditions, such as variation in oxygen tension, in salt concentration, etc., bring about these transmutations, the focal infections can now be looked upon as places "where conditions are favorable for them (bacteria) to acquire the properties which give them a wide range of affinities for various structures (tissues of the host)." Converting hemolytic streptococci into typical *Streptococcus viridans*, *Streptococcus mucosus* and pneumococcus-like organisms seems to indicate transmutability, at least among members of the same family.

Though other examples could be cited, we will merely mention the experience of one of the authors (Stearn, 1923) who found that, after five months culturing in gentian violet broth, four different members of the colon bacillus group exhibited identical cultural characteristics—those of *Bact. coli-communior* A.

Thus it seems that a change in environmental conditions results not only in a change in the chemical constitution of bacteria but also in a corresponding alteration in certain of those characters by which bacteria are classified. In fact, if the previous history of such altered "strains" were not known, they might be classified differently at the end of alteration than at the beginning.

REFERENCES

CLARK 1919 Abs. Bact., **3**, 2.
DAWSON 1919 Jour. Bact., **4**, 142.
EISENBERG 1910 Centralbl. f. Bakt., 1 Abt., Orig., **56**, Heft 3/4.
HISS AND ZINSSER 1916 Textbook of Bacteriology, 3d ed., pp. 36 and 39.
KANTOROWICZ 1909 Münch. med. Wochenschr., **18**, 897.
KELLERMAN AND SCALES 1917 Abs. Bact., **1**, 27.
KLIGLER 1918 Jour. Exper. Med., **27**, 463.
LOEHNIS AND SMITH 1916 Jour. Bact., **1**, 704.
NEIDE 1904 Centralbl. f. Bakt., Abt. 1, **35**, 508.
NIKITIN 1908 Arch. Russ. de Path., **6**, 132 and 162.
REVIS 1913a Proc. Roy. Soc. London, **86**, s. b., 348.
REVIS 1913b Centralbl. f. Bakt., 2 Abt., Jena, **39**, 394.
ROSENOW 1914 Jour. Inf. Dis., **14**, 1.
SCALES 1917 Abs. Bact., **1**, 38.
SHEPPE AND CONSTABLE 1923 Jour. Lab. Clin. Med., **6**, 757.
SMIRNOW 1916 Jour. Bact., **1**, 410.
STEARN, E. W. 1923 Amer. Jour. Pub. Health., **13**, 567.
STEARN AND STEARN 1924 Amer. Jour. Pub. Health., **14**, 409.
WINSLOW, FALK AND CAULFIELD 1923 Jour. Gen. Physiol., **6**, 177.

DIFFERENTIAL STAINING OF LIVING AND DEAD BACTERIAL SPORES

S. A. KOSER AND J. H. MILLS

From the Department of Bacteriology, University of Illinois, Urbana

Received for publication June 5, 1924

Differences in the staining properties of living and dead bacterial cells have been occasionally mentioned in the literature and recently several special staining methods have been suggested to bring out this differentiation more clearly. Seiffert (1922) and Henrici (1923) employed a weak Congo red solution to distinguish living vegetative forms from the dead cells. Cells killed by heat take the stain whereas living cells remain colorless. Burke (1923) has described a difference in the permeability to carbol-fuchsin which is shown by living and heat-killed spores of *Clostridium botulinum*. The dead spores are penetrated by the dye and appear as solid staining forms, whereas unheated cultures show only about 1 to 2 per cent of the solidly stained type while the remainder present an unstained center surrounded by a narrow stained border, the so-called "ring" forms. Evidence is also presented of a definite relationship between penetration by carbol-fuschsin and death of the spore by heat. As a spore suspension of *Clostridium botulinum* is heated the proportion of solid staining spores increases until it becomes 100 per cent and this point is correlated in a general way with the thermal death point as determined by cultivation.

In the present investigation an attempt was made to apply this differential spore stain to the common aerobic spore-formers such as *B. megatherium*, *B. cereus*, *B. subtilis*, etc. The method suggested by Burke (1923) for the spores of *Cl. botulinum* is as follows: Smears are made on glass slides and air dried, heat fixation is omitted. The slides are then stained for two minutes in steaming carbol-fuchsin (1.5 grams basic fuchsin, Grübler,

dissolved in 15 cc. of 95 per cent alcohol is added to 5 cc. of melted phenol crystals in 85 cc. of distilled water), washed, blotted dry and decolorized very briefly with two or three changes of absolute acetone. Wash, blot and counter-stain with Loeffler's methylene blue for two minutes. In our work Coleman and Bell's basic fuchsin was used instead of Grübler's, otherwise the technic of staining followed that outlined by Burke.

Most of our work was done with a laboratory stock culture of *B. megatherium*. Subsequently, the method was extended to other species and the following were used: *B. megatherium* (2 strains), *B. cereus*, *B. subtilis*, *B. ramosus*, *B. mesentericus*, *B. fusiformis*, and *B. terminalis*. Throughout most of the work young agar slant cultures, four to seven days old, were used. There were taken up in sterile water or sterile physiological salt solution and the resultant suspensions were used for preparing smears on glass slides. As a rule the preparations were made and stained immediately. When necessary to kill the spore suspensions by heat they were held in streaming steam in the Arnold sterilizer for one hour. This was found to be sufficient to kill the spores of the various aerobes studied, for subcultures made after the heating were always sterile. Evidently the spores of *B. megatherium* and related types are much less resistant to heat than are those of *Cl. botulinum* since many of the latter will withstand a temperature of 100°C. for several hours (Weiss, 1921; Dickson et al., 1922; Esty and Meyer, 1922; Tanner and McCrea, 1923).

At first an attempt was made to apply the method to *B. megatherium* exactly as it had been outlined for *Cl. botulinum*. However, following the two minutes in steaming carbol-fuchsin it was found that from 50 to 90 per cent of the spores appeared as solid staining forms. Since it is highly improbable that his proportion represented dead spores in the original suspension, it is evident that the combination of carbol-fuchsin with the "steaming" temperature is too severe and that many spores, viable at the outset, were killed or at least were penetrated by the dye during the process. An effort was then made to control the temperature of the staining solution in an endeavor to

find conditions under which young viable spore suspensions would show a large proportion of ring forms and, when killed by heat, would show 100 per cent solid staining forms. This was done by holding the Coplin jar or small beaker containing the carbol-fuchsin solution in a water bath. In this way the temperature of the stain could be adjusted to any desired point and held there. Slides were then immersed in the stain for the proper interval.

The first experiments were designed to determine the effects of temperature and time upon the permeability of *B. megatherium* spores to carbol-fuchsin. In every case the decolorization with

TABLE 1

Effect of time and temperature upon the penetration of spores of B. megatherium by carbol-fuchsin

TEMPERATURE	TIME OF STAINING					
	Two minutes		One minute		Thirty seconds	
	Ring	Solid	Ring	Solid	Ring	Solid
°C.						
25*	98.25†	1.75	99.25	0.75	99.25	0.75
40	94.8	5.2	88.6	11.4	97.6	2.4
50	82.2	17.8	90.5	9.5	95.6	4.4
60	34.9	65.1	78.6	21.4	84.0	16.0
70	3.0	97.0	23.4	76.6	74.9	25.1

* Room temperature.

† Expressed as per cent. At least 400 to 500 spores were counted and the percentage figured upon this basis.

acetone and counterstaining with methylene blue were the same as recommended by Burke. The results are given in table 1. It is quite clear that the penetration of young viable spores by carbol-fuchsin is a function of the time and temperature. As the temperature is raised the proportion of solidly stained spores increases, and at any given temperature the proportion of solid staining forms decreases as the time of staining is shortened. The only discrepancy in the table occurs at 40°C. for one minute. A repetition of this gave 95.8 per cent ring forms and 4.2 per cent solid forms.

Evidently staining for one or two minutes at room temperature best serves the purpose for this organism. The preparations stained for thirty seconds, especially those at the lower temperatures, usually were not as desirable for counting as those given one or two minutes. Many of the ring forms were stained very lightly and for this reason were more difficult to count. Also, some spores exhibited a pink center with a deep red border. In this connection it should be noted that after staining for one or two minutes the spores almost invariably fell into the two sharply defined groups. The solidly stained spores were a deep red throughout and stood out in decided contrast to the pink oval

TABLE 2

Proportions of ring and solid stained spores in unheated and heated suspensions of B. megatherium

	STAINED AT ROOM TEMPERATURE					
	Two minutes		One minute		Thirty seconds	
	Ring	Solid	Ring	Solid	Ring	Solid
Unheated	97.8*	2.2	99.0	1.0	99.3	0.7
Heated	0.0	100.0	0.0	100.0	0.0	100.0

* Expressed as per cent. Four to five hundred spores were counted and the percentage figured upon this basis.

"ring" forms with the colorless center. Very few intermediate stages were seen. The colored outline of the ring forms varied somewhat in thickness, but as the colorless center was always in evidence this caused no confusion in counting the preparations. A few spores were seen which showed a pink center surrounded by a deep red ring. These were encountered infrequently and were classed as solid staining forms. The picture presented by *B. megatherium* after staining by this method for one or two minutes at room temperature is quite similar to that described by Burke (1923) for *Cl. botulinum* after staining two minutes with steaming carbol-fuchsin. Evidently the penetration of stain to the interior of the spore occurs very quickly, since in the graded series of temperatures shown in table 1 very few intermediate forms were seen as the proportion of solidly stained spores increased.

A comparison of unheated and heat-killed *B. megatherium* spores is given in table 2. The decided contrast in the results of the differential stain is quite striking and needs little comment.

TABLE 3

Differential counts on mixtures of living and heat-killed spore suspensions of B. megatherium

PROPORTION OF SUSPENSION BY VOLUME	DIFFERENTIATION BY STAINING TWO MINUTES AT ROOM TEMPERATURE	
	Per cent of solid forms	Per cent of ring forms
3 heated : 1 living	79.6	20.4
	80.4	19.6
	76.6	23.4
1 heated : 1 living	49.97	50.03
	48.3	51.7
	51.4	48.6
	49.2	50.8
	54.9	45.1
1 heated : 2 living	33.65	66.35
	31.8	68.2
	32.4	67.6
1 heated : 3 living	28.1	71.9
	23.1	76.9
	27.4	72.6
1 heated : 5 living	9.33	90.67
Controls:		
Unheated	1.5	98.5
	1.7	98.3
	2.5	97.5
All heated	100.0	0.0
	100.0	0.0

A total of 500 to 1000 spores was counted in each preparation.

The killed spores invariably were penetrated by the dye and such preparations showed 100 per cent solid staining forms.

To further control the accuracy of this method definite mixtures of living and heated spores of *B. megatherium* were pre-

pared and a differential count was then made on the basis of the stain. Spore suspensions were prepared from agar slants four or five days old and part of each suspension was killed by heating for one hour in the Arnold sterilizer. The living and heat-killed spore suspension were then mixed in various proportions, well shaken and a loopful of each mixture removed to a slide, air-dried and stained with carbol-fuchsin for two minutes at room temperature. The decolorization with absolute acetone and the methylene blue counterstain were applied as usual. Several different preparations of each mixture were made and counted. The results of this experiment are given in table 3. By the use of the differential stain it was possible to determine fairly accurately the proportion of heat killed spores in the various mixtures. The appearance of the solidly stained spores and the ring forms presented a very decided contrast and no difficulty was encountered in distinguishing them.

The results of the differential stain were next compared with the thermal death point as determined by cultivation. The same strain of *B. megatherium* was used for these experiments. To avoid the complication of numerous vegetative cells in the spore suspensions which might cause a decrease in the plate count after heating, agar slant cultures from two to three weeks old were employed. These were taken up in sterile water after the usual procedure. The particular culture of the *B. megatherium* used in these experiments formed spores very readily and stains made from suspensions of the two to three weeks old agar slants revealed very few vegetative cells, not enough to cause any appreciable decrease in the plate count after the first interval of heating. The spore suspensions were divided among a number of narrow thin-walled test tubes and heated in a DeKhotinsky oil bath for varying lengths of time. At each time interval a tube was removed from the bath and cooled by immediately placing in cold water. Plates were then made from each tube to determine the number surviving and at the same time a drop of the material was placed on a slide, air-dried and stained. The percentage of spores killed, as determined by the plate count, could then be compared with the per cent of solid staining forms as brought

out by the differential stain. A number of experiments were run at 90° and at 100°C. Two typical series of results are given in table 4. At the outset of the experiment the unheated spore suspensions always showed a small proportion of solid forms. This is in harmony with previous work. As the heating progresses and the spores are killed, it is noteworthy that the proportion of

TABLE 4

Comparison of death rates of spores of B. megatherium as determined by plating and by the differential stain

TEMPERATURE AND TIME HEATED	PLATE COUNT PER CUBIC CENTIMETER	PER CENT KILLED AS SHOWN BY	
		Plate count	Differential stain (per cent of solid forms)
Original (unheated)	47,000,000		3.9
Heated 90°C., 2½ minutes	12,000,000	74.5	11.7
Heated 90°C., 5 minutes	2,600,000	94.5	19.6
Heated 90°C., 7½ minutes	365,000	99.22	32.9
Heated 90°C., 10 minutes	85,000	99.82	53.1
Heated 90°C., 15 minutes	9,500	99.98	83.7
Heated 90°C., 20 minutes	1,800	99.99+	92.5
Heated 90°C., 30 minutes	60	99.999+	99.4
Heated 90°C., 45 minutes	3	100.0−	100.0
Heated 90°C., 60 minutes	0	100.0	100.0
Original (unheated)	26,000,000		2.56
Heated 100°C., 1 minute	18,800,000	27.7	2.5
Heated 100°C., 2 minutes	6,500,000	75.0	6.6
Heated 100°C., 3 minutes	900,000	96.5	21.2
Heated 100°C., 4 minutes	180,000	99.3	42.2
Heated 100°C., 5 minutes	2,000	99.992	70.6
Heated 100°C., 7½ minutes	1	100.−	100.0
Heated 100°C., 10 minutes	0	100.0	100.0
Heated 100°C., 15 minutes	0	100.0	100.0

solidly stained spores is always lower than the per cent killed as shown by the plate count. The discrepancy between these two methods of estimating the proportion of spores killed is greatest near the outset of the heating process and then as the point is approached at which all spores are destroyed the per cent of solid forms increases rapidly. Finally, when viable spores can no longer be detected by cultivation, the solid

staining forms constitute 100 per cent of the preparations and the two methods then check very accurately. Similar results were obtained upon several repetitions of this experiment and it is believed that errors in technic cannot account for the results obtained. It seems evident that during the process of heating,

TABLE 5

The differential staining method applied to various aerobic spore-formers

	STAINED AT ROOM TEMPERATURE			
	Two minutes		One minute	
	Ring	Solid	Ring	Solid
B. megatherium (2nd culture):				
Unheated	94.3	5.7	95.8	4.2
Heated	0.0	100.0	0.0	100.0
B. cereus:				
Unheated	96.5	3.5	98.9	1.1
Heated	0.0	100.0	0.25	99.75
B. subtilis:				
Unheated	95.0	5.0	96.25	3.75
Heated	0.0	100.0	0.0	100.0
B. mesentericus:				
Unheated	95.1	4.9	96.3	3.7
Heated	0.0	100.0	0.0	100.0
B. ramosus:				
Unheated	97.0	3.0	98.7	1.3
Heated	0.0	100.0	0.0	100.0
B. terminalis:				
Unheated	86.6	13.4	89.9	10.1
Heated	15.2	84.8	17.9	82.1
B. fusiformis: Unheated, Heated	Practically all ring forms, these take the counterstain and appear blue. No differentiation between heated and living			

the point at which spores are rendered incapable of germination and the point at which they are penetrated by the stain are not the same. Penetration by the stain always occurs just after the spore has been killed, or at least rendered unable to germinate when transferred to suitable media. In this connection it should be mentioned that Burke (1923), in her work with *Clostridium botu-*

linum, obtained evidence that loss of viability slightly preceded complete dye penetration or solid staining. This conclusion was based upon a comparison of the maximum survival of C. *botulinum* spores heated in broth and the corresponding percentages of solid staining forms heated in the same medium.

The results of the spore stain when applied to several miscellaneous types of aerobic spore-formers are shown in table 5. In these cases agar slants one week old were used as the source of spores. Two staining procedures were tried; in one the carbol-fuchsin was applied for one minute and in the other for two minutes. The usual procedure of decolorization and counterstaining was followed. With most of the organisms the stain served to differentiate very nicely between the unheated and heat-killed spore suspensions. The heated suspensions showed 100 per cent solid staining spores whereas the preparations made from unheated spores showed only a few of the solid type and consisted very largely of the ring forms. Two cultures gave unsatisfactory results. *Bacillus terminalis* did not show a marked contrast between the unheated and heated preparations and in addition many of the ring forms took the counterstain and appeared blue instead of red. The results secured with *B. fusiformis* were even more unsatisfactory for there appeared to be no difference at all between the heated and unheated spores. None of the heat-killed spores took the carbol-fuchsin, but all spores, both heated and unheated, took the counterstain and appeared as blue ring forms. It is possible that a modification of the staining technic might be found which would give satisfactory results with these last two cultures. This was not investigated however. It should be pointed out that each species, or even each strain within the species, may present a different degree of permeability and in each case the time and temperature must be chosen which serves to bring out the best differentiation. Evidently this is true even with the highly resistant *Cl. botulinum* spores for Burke (1923) states that the period of immersion in the carbol-fuchsin should vary slightly with different strains of the organism.

It will be seen that in the unheated suspensions, although the ring forms greatly predominated they never reached 100 per cent. The preparations of all of the organisms studied invariably showed a small proportion of solid staining spores, even when young spores from three to four day agar slants were used. This number varied usually from 1 to 5 per cent with an occasional higher figure. The point may be raised as to whether the staining method is entirely accurate in this respect, since these solid forms may really not represent dead spores. This is largely conjecture at the present time since little is known about the death-rate of spores of the various species under different conditions and at different ages of the culture. In this connection the work reported by Swann (1924) is especially interesting. Upon the basis of microscopic observations of the germination time of spores of *B. anthracis*, he states that 5 per cent of young anthrax spores (two to three days old) and about 55 per cent of old dried spores (one year old) are dead. Also, it appears that even young actively growing vegetative cells may have a certain death-rate, for Wilson (1922) has reported that during the period of logarithmic growth of *Bact. suipestifer* the percentage of viable organisms seldom rose above 90 per cent of the total and frequently it fell considerably below this figure. Thus the available evidence indicates that we may expect a small proportion of dead cells, both spore and vegetative forms. The results secured by the differential spore stain are in harmony with this viewpoint.

Finally, it should be emphasized that thus far the stain has been applied only to spores killed by heat. A few miscellaneous observations were made upon *B. megatherium* spores after they had been suspended in a 0.1 per cent mercuric chloride solution until no growth occurred when transferred to suitable media. These spores were not penetrated by the stain and appeared as ring forms. There is the possibility of course that they were not killed but merely prevented from germinating by the disinfectant. In any event the results of staining were not in agreement with the results of cultivation. The extension of the staining method to spores killed by agencies other than moist heat was not investigated further at this time.

SUMMARY

The staining method suggested by Burke for differentiating the living and dead spores of *Clostridium botulinum* was modified and applied to several miscellaneous aerobic spore-formers. With most of these a good differentiation was obtained, though two cultures gave unsatisfactory results.

In additional investigations a strain of *B. megatherium* was used. It was found that penetration of the spores by the dye is dependent upon the time and temperature at which the stain-solution is applied. One or two minutes in carbol-fuchsin at room temperature gave the best results with the organisms studied. Spores killed by heat are penetrated by the stain and appear as solid staining forms, whereas unheated spores from young cultures show a very large proportion of ring forms with only a low percentage of the solidly stained forms.

The method appears to be reliable and permitted the determination of the percentage of heated and living spores in mixtures of known proportions.

There is evidence that during the process of heating, the point at which spores are rendered incapable of germination and the point at which they are penetrated by the dye are not the same. Apparently penetration by the stain occurs just after the spore has been killed or at least rendered unable to germinate when transferred to suitable media. However, after heating sufficiently to kill all spores in the suspension the proportion of solidly stained spores was uniformly 100 per cent and agreed with the negative results secured upon cultivation.

REFERENCES

BURKE, G. S. 1923 Studies on the thermal death time of spores of *Clostridium botulinum*. 2. The differential staining of living and dead spores. Jour. Inf. Diseases, **32**, 433–438.

DICKSON ET AL. 1922 Studies on the thermal death time of spores of *Clostridium botulinum*. Preliminary report. Jour. Amer. Med. Assoc., **79**, 1239–1240.

ESTY, J. R., AND MEYER, K. F. 1922 The heat resistance of the spores of *B. botulinus* and allied anaerobes. Jour. Inf. Diseases, **31**, 650–664.

HENRICI, A. T. 1923 Differential counting of living and dead cells of bacteria. Proc. Soc. Exper. Biol. and Med., **20**, 293–295.

SEIFFERT, W. 1922 Vergleichende Färbeversuche an lebenden und toten Bakterien. Centralbl. f. Bakt., Abt. I, Orig., **88**, 151–158.

SWANN, M. B. R. 1924 On the germination period and mortality of the spores of *Bacillus anthracis*. Jour. Path. and Bact., **27**, 130–134.

TANNER, F. W., AND MCCREA, F. D. 1923. *Clostridium botulinum*. IV. Resistance of spores to moist heat. Jour. Bact., **8**, 269–276.

WEISS, H. 1921. The heat resistance of spores with special reference to the spores of *B. botulinus*. Jour. Inf. Diseases, **28**, 70–92.

WILSON, G. S. 1922 The proportion of viable bacteria in young cultures with especial reference to the technic employed in counting. Jour. Bact., **7**, 405–446.

ANAEROBIC SPORULATING THERMOPHILES

SOME OBSERVATIONS ON A NEW GROUP OF BACTERIA

S. R. DAMON AND W. A. FEIRER

From the Department of Bacteriology of the School of Hygiene and Public Health, The Johns Hopkins University, Baltimore

Received for publication June 25, 1924

HISTORICAL RÉSUMÉ

In the literature relating to the bacteria growing at high temperatures there are but three references, so far as we are aware, to thermophilic anaerobes. The earliest reference to bacteria of this type is found in the work of Oprescu (1898) who described three species of obligate anaerobes, although there may be a reasonable doubt whether these species were really anaerobic. Somewhat later Benignetti (1905) isolated a single species of a thermophilic bacillus from water, which could be cultivated anaerobically. And finally, in 1906, Bardou (1906) reported the isolation from sewage of four strains of facultative anaerobes that grew equally well at 18° and 60°C. These organisms were characteristically putrefactive at either temperature. In contrast to the organisms that we will describe, it should be noted that the strains isolated by Bardou had the faculty of growing in the presence of atmospheric oxygen.

SOURCE OF THE CULTURES AND METHODS EMPLOYED

The organisms to be described in this paper were isolated from well-rotted horse manure. The method of isolation was the same in all cases. A suspension of the feces was made in sterile water and deep infusion agar shake tubes inoculated with from 1 to 3 drops of suitable dilutions of this suspension, following the technique of Veillon-Zuber (1898). These tubes were incubated from two to five days at 55°C. and the single isolated colonies

cut out, sterilized by the application of tincture of iodine and transferred to the cooked meat medium of Holman (1919) which had previously been boiled for fifteen minutes in order to expel the dissolved oxygen. At this point melted vaseline to a depth of 1.5 to 2 cm. was poured over the meat, as it would seem that anaerobiosis at thermophilic temperatures is somewhat aided by this means. In the majority of cases good growth is observed in the meat tubes after forty-eight hours' incubation although this is confined to the immediate vicinity of the meat particles and the supernatant broth remains clear. More profuse growth in the meat tubes was obtained by the addition of about 5 cc. of sterile aqueous infusion of horse manure to the medium.

From the meat tubes, which were used as stock cultures, transfers were made, using a Pasteur pipette, to the various media. In all cases the growth of the organisms was tested aerobically and anaerobically on infusion agar slants. The anaerobic tests were made in a McIntosh and Fildes (1916) jar. The organisms were also planted on the usual anaerobic media, i.e., egg cube, alkaline egg, litmus milk, brain, and lactose, sucrose, glucose, maltose, starch, glycerol, inulin, and mannitol broth. Only a meager growth was observed in these media and there was no visible reaction.

Throughout all our work veal infusion media were used for both aerobic and anaerobic cultivation. In the cases where sugar free meat was used it was freed from muscle sugar by inoculation with *B. coli-communior,* incubated eighteen hours at 37°C., tubed and sterilized.

The solid media in which a carbohydrate was included were prepared from 1.5 per cent veal infusion agar by adding 1 per cent of the carbohydrate and 1 per cent Andrade indicator.

The test for nitrate reduction was performed by incubating veal infusion nitrate broth for forty-eight hours and then testing for the presence of nitrite by the addition of a'pha-naphthylamin and sulphanilic acid.

Hydrogen sulphide production was observed by the formation of lead sulphide in veal infusion agar shake tubes to which 0.1 per cent basic lead acetate had been added.

The production of indol was tested for by the methods of Ehrlich and Salkowsky and the vanillin test.

The thermal resistance of the organisms described herein was determined by submitting the broth from a meat culture which had been incubated for forty-eight hours at 60°C, to heating for ten minutes at 60°, 70°, 80°, 90°, 100°, 110° and 120°C. The cultures were immersed and heated in a DeKhotinsky oil bath. After the period of heating subcultures were made in veal infusion agar shake tubes and incubated at 55°C. for five days.

MORPHOLOGICAL AND CULTURAL OBSERVATIONS

In the description of the following organisms the generic name "Clostridium" has been adopted as suggested by the Society of American Bacteriologists for all anaerobic spore-forming bacilli. The selection of a term to indicate the species has been dictated by the effort to incorporate the thermophilic character and the most outstanding feature of the organism in a single word.

During this investigation many thermophilic cultures have been isolated which were facultative anaerobes. A report on the cultural characteristics of these organisms will be made in a subsequent paper. The detailed descriptions given in this paper apply only to organisms conclusively demonstrated to be obligate anaerobic thermophiles. A few of the cultures show slight growth at 37°C. as well as at 55°C. but in general their characteristic reactions are best observed at the higher temperature.

Clostridium thermoputrificum

This organism is an obligate anaerobic thermophile isolated from fecal material derived from the horse. It undoubtedly represents a group of organisms having considerable putrefactive power and capable of exhibiting its reactions at 37°C. as well as at 55°C.

Morphology. Uniform short rods with homogeneous protoplasm and rounded ends. The average dimensions of the rods taken from a forty-eight hour chopped meat culture, incubated at 55°C. are 5 by 2.5 microns.

Staining properties. The organisms are gram-positive in forty-eight hour meat cultures. They lose their ability to retain the gentian violet after five days incubation.

Motility. Non-motile in meat cultures examined every two hours from four to forty-eight hours incubation. No flagella demonstrable.

Spore-formation. Spores are observed in forty-eight hour meat cultures. They are terminal and oval, and bulge the rod slightly.

Agar shake colonies. Single colonies in 2 per cent veal infusion agar are small, discus shaped, with well defined margins as seen under the low power.

Cooked meat medium. After two days incubation at from 37° to 55°C. gas bubbles are noted throughout the medium. At 37°C. the vaseline cap is raised about 1 cm. at the end of four days incubation. Upon penetration of the vaseline cap a strong putrefactive odor is noted, and at the end of four days there is a definite proteolysis as indicated by the digestion of the meat. There is no change in the color of the medium.

Sugar-free cooked meat shows reddening and a hazy diffuse growth throughout the medium.

In both media the broth above the meat remains clear except for a narrow zone of turbidity immediately above the meat particles.

Fermentation reaction. Lactose, glucose, starch. Growth at from 37° to 55°C. but no acid or gas produced.

Maltose, glycerol, sucrose, mannitol and inulin. Growth, with a marked production of gas, but no acid indicated at 37° to 55°C. with Andrade indicator.

Lead acetate infusion agar. Hydrogen sulphide produced as evidence by a black coloration of the medium at 37° to 55°C.

This culture does not reduce nitrates; it does not have any action on gelatin, nor does it produce indol.

Clostridium thermoaerogenes

This organism is an obligate anaerobe growing only at 45° to 55°C. It was isolated from horse manure. A number of cultures were obtained which gave identical reactions as indicated below. None of the cultures exhibited any putrefactive action. Gas, however, was produced in certain of the carbohydrate media, but no acid was formed.

Morphology. A uniform medium-sized rod with granular protoplasm and rounded ends. The average dimensions of the rods in a forty-eight hour meat culture incubated at 55°C. are 7 by 1.8 microns.

Staining properties. Gram-negative in forty-eight hour meat cultures.

Motility. Non-motile in meat cultures examined every two hours after four to forty-eight hours. Incubation at 55°C. No flagella demonstrable.

Spore-formation. Spores observed in forty-eight hour meat cultures incubated at 60°C. They are terminal, oval, and bulge the rod slightly.

Agar shake colonies. Single colonies in 2 per cent veal infusion agar are round and have well defined margins under the low power.

Cooked meat medium. After two days incubation at 55°C. gas bubbles are noted throughout the medium. There is no change in the color of the meat or sign of digestion.

Sugar-free cooked meat shows a reddening and a hazy diffuse growth throughout the meat particles. A narrow zone of broth above the meat is turbid, above this the broth is clear.

Fermentation reactions. Lactose, glycerol, sucrose, mannitol and glucose show growth but no acid or gas is formed.

Maltose and inulin: Gas is produced, but no acid is indicated by the Andrade. Starch: Gas and acid produced.

Nitrate reduction. Nitrates are reduced to nitrites after forty-eight hours incubation at 55°C. These cultures show no reaction on lead acetate or gelatin and do not produce indol.

One culture was isolated which was identical with *C. thermoaerogenes* except that it produced acid in inulin.

Three cultures were isolated which were morphologically similar and exhibited the same reactions at 55°C. as the above organism but also had the ability to grow at 37°C. without any alteration of the media.

Clostridium thermoacidophilus

This organism is an obligate anaerobic thermophile isolated from horse manure. A number of cultures were isolated which gave acid on some carbohydrates and acid and gas on others. None of the cultures exhibited any digestion of the meat.

Morphology. A long rod with granular protoplasm and rounded ends. The average dimensions of the rods in a forty-eight hour meat culture incubated at 55°C. are 10.8 by 1.2 microns.

Staining properties. Gram-positive in a forty-eight hour meat culture. The organisms lose their ability to retain the gentian violet after five days incubation.

Motility. Non-motile in meat cultures examined every two hours after four to forty-eight hours incubation at 55°C. No flagella were demonstrable.

Spore formation. Spores observed in forty-eight hour meat cultures incubated at 60°C. They are terminal, oval, and bulge the rod slightly.

Agar shake colonies. In 2 per cent veal infusion agar shake tubes single colonies are like a burr; no definite margin could be seen under low power.

Cooked meat medium. After two days incubation at 55°C. gas bubbles are noted throughout the medium. There is no change in color and no digestion is noticed in the meat.

Sugar-free meat medium shows a reddening and a hazy diffuse growth throughout the meat particles. The broth is clear except for a small zone just adjacent to the meat.

Fermentation reactions. At 55°C. Lactose, maltose and glucose show growth but no acid or gas is observed.

No gas is produced in glycerol, starch and sucrose, but acid is observed. Gas and acid are seen in mannitol and inulin.

Nitrate reduction. Nitrates are reduced to nitrites in forty-eight hours. These cultures show no reaction on lead acetate or gelatin, and do not produce indol.

One culture was isolated which was morphologically similar to the above strain and exhibited the same carbohydrate reactions at 55°C., but did not reduce nitrates.

Two cultures were isolated which were similar to *C. thermoacidophilus* but grew at 37°C. with no apparent reactions although one of them has the power to liquefy gelatin at 55°C.

Clostridium thermochainus

This organism is an obligate anaerobic thermophile isolated from horse feces. Three cultures were isolated which produced acid from starch and sucrose. None of the cultures exhibited any visible action on meat.

Morphology. A uniform medium rod with granular protoplasm and slightly rounded ends. The rods may appear singly or in chains of from 3 to 6 organisms. The average dimensions of the single rods in a forty-eight hour meat culture incubated at 55°C. are 7 by 1.8 microns.

Staining properties. Gram-positive in a forty-eight hour meat culture. After five days incubation the organisms lose their ability to retain the gentian violet.

Motility. The cultures are non-motile in meat cultures examined every two hours after four to forty-eight hours incubation at 55°C. No flagella are demonstrable.

Spore formation. Spores observed in forty-eight hour meat cultures incubated at 60°C. They are terminal, oval and bulge the rod slightly.

Agar shake colonies. In 2 per cent veal infusion agar single colonies are round with well-defined margins under low power magnification.

Cooked meat media. After two days incubation at 55°C. gas bubbles are noted throughout the medium. There is no change in color or digestion noticed in the meat.

Sugar-free meat medium shows a reddening and a hazy diffuse growth throughout the meat particles. The broth is clear above the meat.

Fermentation reactions at 55°C. Maltose, glycerol, lactose, mannitol, inulin and glucose show growth but no acid with Andrade indicator. No gas bubbles are seen in the medium.

Starch and sucrose show slight acid production with Andrade indicator, but no gas is observed.

Nitrates are reduced to nitrites after two days incubation at 55°C. These cultures show no reaction on gelatin or on lead acetate, and do not produce indol.

TABLE 1

Carbohydrate reactions

CULTURES	INCUBATION TEMPERATURE	MALTOSE		GLYCEROL		STARCH		SUCROSE		LACTOSE		MANNITOL		INULIN		GLUCOSE	
		Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas
	°c.																
C. thermoputrificum	55		+		+				+				+		+		
	37		+		+				+				+		+		
C. thermoaerogenes	55		+			+	+								+		
	37																
C. thermoacidophilus	55			+		+		+				+	+	+	+		
	37																
C. thermochainus	55					+		+									
	37																

It will be seen that, with the exception of *C. thermoputrificum*, all the species are obligate thermophiles, and that, with this exception, their fermentative activity is observed at 55°C. only.

TABLE 2

Morphology, size, colony formation and thermal resistance

CULTURES	MORPHOLOGY	SIZE IN MICRONS	COLONY FORMATION IN 2 PER CENT AGAR SHAKE TUBES	THERMAL RESISTANCE
		μ		
C. thermoputrificum	Short rod, Gram-postive, with terminal spore	5 x 2.4	Small discus-shaped	Thermal death point between 110° and 120°C. ten minutes
C. thermoaerogenes	Medium, Gram-negative, granular, with terminal spore	7 x 1.8	Round, sharply outlined	Thermal death point between 110° and 120°C. for ten minutes
C. thermoacidophilus	Long rod, Gram-positive, granular, with terminal spore	10.8 x 1.2	Large, fuzzy, burr-like	Thermal death point between 110° and 120°C. for ten minutes
C. thermochainus	Medium, Gram-positive, granular, chains, terminal spore	7 x 1.8	Round, sharply outlined	Thermal death point between 110° and 120°C. for ten minutes

DISCUSSION

The organisms described by Oprescu, Benignetti, and Bardou as thermophilic anaerobes have all been observed to have the faculty of growing in the presence of atmospheric oxygen. In contrast to this the species described in this paper grow only under conditions of strict anaerobiosis. This fact is demonstrated by the observation of a clear zone, in which no colonies are seen, to a depth of 2 to 3 cm. in the infusion agar shake tubes.

It is true that certain cultures were seen to grow under anaerobic conditions at 37°C. but exhibited no reaction in any of the media at this temperature. An exception must be made in the case of *C. thermoputrificum*, however, as this organism exhibits all its cultural reactions at 37° as well as at 55°C.

A series of cultures incubated at 65°C. for five days showed no growth, demonstrating that the limiting temperature for growth is between 60° and 65°C.

In the table of carbohydrate reaction it is seen that in two species gas is produced from maltose, glycerol, sucrose, mannitol, and inulin, and that no acid could be detected with Andrade indicator. A possible explanation for this phenomenon is that the little acid which was produced may have been neutralized by the buffer in the medium in those cases where gas but no acid was observed. It is suggested also that a possible explanation may lie in the fact that the unattacked carbohydrate present stimulated the activity of the organisms to the point where protein fermentation took place.

With the exception of *C. thermoputrificum*, the reactions of all the cultures were meager, although perfectly definite. In some of the cultures where gas production was noted there were only a few gas bubbles produced in the media.

Aerobic and anaerobic control tubes were run with each series of media at 37° and 55°C.

No growth, aerobically or anaerobically, was observed in any of the cultures at room temperature.

In the determination of the heat resistance of the spores it was found that some viable spores were found after heating at 110°C. for ten minutes, but no growth could be seen in subcultures in tubes heated at 120°C. for ten minutes even after five days incubation.

The pathogenicity of all the cultures was tested by subcutaneous and intraperitoneal inoculation in guinea pigs, using 0.5 per cent of the body weight of inoculum. No pathogenic action was observed in any of the cultures.

CONCLUSIONS

1. A group of obligate anaerobic thermophiles has been described for the first time.

2. The species described belong to the genus Clostridium by virtue of their anaerobic spore-forming character.

3. One culture has been isolated which has an optimum temperature range of from 37° to 55°C. and which is actively proteolytic throughout this range.

4. The limiting temperature of growth for these cultures is between 60° and 65°C.

5. The thermal death point of these cultures lies between 110° and 120°C.

6. None of the cultures are pathogenic for guinea-pigs.

REFERENCES

BARDOU 1906 Thèse, Lille.
BENIGNETTI, DIÉGO 1905 Revistá d'Igiene e sanitá publica,
HOLMAN 1919 Jour. Bact., **4**, 149.
MCINTOSH AND FILDES 1916 Lancet, **1**, 768.
OPRESCU 1898 Arch. f. Hyg., **33**, 164; Hyg. Rundsch., **8**, 107.
VEILLON-ZUBER 1898 Arch. de Med., **10**, 517.

ON THE SURVIVAL OF BACTERIUM TYPHOSUM INTRAPERITONEALLY IMPLANTED IN COLLODION SACS

FREDERICK L. GATES

From the Laboratories of the Rockefeller Institute for Medical Research, New York City

Received for publication June 25, 1924

In the course of certain experiments on the permeability of collodion membranes, it was found that the body fluids could furnish diffusible nutrient substances to bacteria in intraperitoneally implanted collodion sacs (Gates, 1921). Conversely, although sterile collodion sacs were practically inert, an active proliferative cell reaction occurred around sacs containing living bacteria, thus demonstrating the passage of irritating bacterial products in the opposite direction. These reactions were of interest in relation to the survival of bacteria *in vivo* and to antibody formation as a result of chronic local infection. The reaction of the body to a localized infection with *Bacterium typhosum*, enclosed in collodion sacs, was therefore observed in a series of 8 rabbits, 4 of which were killed for examination after from twenty-four to forty-five days, and the others after a survival of from fifteen to nineteen months.

EXPERIMENTAL

Under ether anesthesia, each rabbit was implanted intraperitoneally with 3 or 4 collodion sacs of about 5 cc. capacity (Gates, 1921), containing distilled water or 0.85 per cent salt solution and inoculated with a strain of *Bact. typhosum* originally obtained by Dr. C. G. Bull from an ampoule of Besredka's sensitized typhoid vaccine. Two of the rabbits carried uninoculated control sacs also.

The effects of implantation on the host

The presence of the sacs had no observable effect upon the health of the animals. No variations from normal were observed in temperature, weight, or appetite. Shortly after implantation, sacs containing living microörganisms became covered with a mantle of fibrin and wandering cells. Later this exudate became organized and replaced by ingrowth of connective tissue cells and vascular elements until the sac, or group of sacs, formed the center of a dense pedunculated mass of fibrous tissue with vascular walls from 1 to 3 mm. in thickness. There was never any evidence of intestinal obstruction or other interference with function due to the presence of these masses. They were usually attached to the omentum. In all the rabbits of this series the abdominal organs appeared normal, or showed only the lesions of coccidiosis which are frequently encountered in stock animals.

Collodion sacs do not withstand intraperitoneal conditions indefinitely. The material gradually deteriorates, losing its transparency and elasticity, so that after a time the sacs break and disintegrate. After twenty-four days, 8 sacs in 2 rabbits were found intact, as were 3 sacs in another rabbit after 41 days incubation. After forty-five days, however, 2 of the 3 sacs in a fourth rabbit were broken and all of the sacs in the 4 rabbits examined more than a year later had collapsed. It seems probable that in these animals the sacs did not remain intact longer than one or two months and thereafter served only as the focus of a localized infection. At the time that the sacs broke they were undoubtedly already surrounded by a capsule which prevented a wide dissemination of the liberated organisms. At autopsy no other foci of typhoid infection were discovered. Cultures of the blood, urine, and bile were negative for *Bacterium typhosum*.

Examination of sac contents after intraperitoneal incubation

In the shorter experiments (twenty-four or forty-one days) when the sacs were still intact, they were surrounded by a small amount of sterile seropurulent fluid. The sacs themselves were

filled with a milky material which separated, on standing, into a cloudy, straw-colored fluid of pH 7.4 to 7.6 on the Sörensen scale, and a cream-colored sediment whose volume was from $\frac{1}{4}$ to $\frac{1}{3}$ of the whole. This heavy deposit consisted of amorphous débris and a variety of bizarre-shaped Gram-negative bodies, coccoidal and bacillary, which took bacterial stains with all degrees of intensity and often were stained more heavily at the poles. Some forms were typical of *Bacterium typhosum*. Larger, oval and spherical, deeply stained bodies such as are often called "involution forms" were also observed. The amount of this material was far in excess of what is ever formed in old cultures of *Bacterium typhosum*, *in vitro*. Multiplication had proceeded long after growth would have ceased in an artificial medium. Obviously the continued growth was due to a persisting diffusion of fresh nutrient material through the sac wall. From the contents of these sacs, intact after twenty-four or forty-one days incubation, pure cultures of typical *Bacterium typhosum* were obtained.

The filtered supernatant fluid from two sacs which had been in the peritoneal cavity of Rabbit 5 for forty-one days proved somewhat toxic for a normal rabbit only when injected intravenously in a dose of 5 cc. It caused a transient rise in temperature and a temporary depression.

The sacs which had broken during forty-five days incubation contained a milky fluid and sediment made up of bacterial débris and flakes of pus. Films showed many degenerated leucocytes and some typical monocytes and polynuclear cells. No phagocytosis was observed. The unbroken sac contained the same bacterial forms but no leucocytes. The contents of the sacs produced confluent growths of *Bacterium typhosum* in pure culture.

The next observations were made on the 4 rabbits which were allowed to survive for periods of fifteen to nineteen months. The findings were practically identical throughout. A section of the dense, tough capsules of fibrous tissue disclosed a mass of thick, white, creamy or cheesy material containing the broken remnants of a collodion sac. This material consisted of amorphous, diffusely and irregularly staining débris, in which no wandering cells could

be identified. Many "shadows" of bacillary shape made blank spaces in the background. Recognizable stained organisms were rarely encountered. Nevertheless, each pocket in the capsular masses yielded a luxuriant, confluent growth of *Bacterium typhosum*. Some of the sacs had been inoculated with cultures derived from sacs of the earlier series and so represented a second period of incubation *in vivo*. Cultures from the capsules were compared with the original strain, kept on nutrient agar in the laboratory. Even after long periods of survival in the animal body in more or less intimate contact with wandering cells and tissue fluids, no change whatever in the morphology, staining reactions, antigenic properties or functional activity of *Bacterium typhosum* could be observed.

Zinsser and Raymond (1921) recently published a note which is of interest in this connection. Celloidin capsules made on candy cores and emptied by dialysis were punctured with a needle and inoculated with streptococci in agar. The puncture hole was left open. Rabbits harboring such celloidin sacs in the peritoneal cavity usually lived for months. Some gradually emaciated, others developed agglutinins. After four months the capsules were found to contain living streptococci. The organisms recovered were morphologically and culturally identical with those implanted. The authors consider that their experiments furnish "some evidence, at least, against the mutations of streptococci in the animal body."

Antibody production

The serum of the rabbits tested agglutinated *Bacterium typhosum* in dilutions of from 1:32 to 1:100 after the twelfth to sixteenth day, when the sacs were certainly or supposedly intact. On the forty-first day when 1 rabbit was killed and found to harbor unbroken sacs, the agglutinin titer of the animal's serum was 1:160. Apparently there was some passage of agglutinogen through the sac walls. After the sacs had almost certainly broken, higher titers were found in the serum of the rabbits which were allowed to survive. Even so, the antibody formation was slight in comparison to that readily obtained with small doses of

Bacterium typhosum injected intravenously. The highest titer observed was 1:800 in the serum of one rabbit six months after the sac implantation. The titers of serum specimens from the other 3 rabbits ranged from 1:25 to 1:500, six to nine months after operation. Thereafter the agglutinin content of the sera fell off gradually until the tests made after a year showed agglutination only in serum dilutions of 1:4 to 1:8. Such figures indicate that practically no agglutinogen was being produced in the capsules, or that the foreign material was so completely walled off by the fibrous tissue that no absorption of agglutinogen was taking place.

In 1901 McCrae (1901) reported somewhat similar results from the intraperitoneal implantation of bacteria of the colon group contained in celloidin capsules. In one instance an agglutination titer of 1:1000 was obtained against *Bacterium enteritidis* on the twenty-first day. After the capsule had been removed on the twenty-sixth day the serum titer had dropped to 1:500 by the thirty-first day. McCrae concluded that agglutinins in the serum are strictly associated with the existence of bacteria in a living state in the body, and noted that his observations might explain long-continued reactions in the serum of persons who had recovered from typhoid fever. He referred to the persistence of typhoid bacilli in the gall bladder and in abscesses, which proves that the microörganisms may survive for long periods "either lying latent or proliferating very slowly in some one or other region of the body."

SUMMARY

Eight rabbits, intraperitoneally implanted with collodion sacs containing *Bacterium typhosum* in salt solution or distilled water, furnished nutrient materials to the organisms which promoted luxuriant growth and maintained the bacilli in a viable condition for periods up to nineteen months, the longest interval tested. The bacteria developed in many bizarre shapes and sizes, but these forms were either not viable, or reverted to normal on transfer to a more favorable medium. Even after a sojourn of many months *in vivo*, no permanent change in morphological, cultural, or antigenic properties could be detected.

The rabbits themselves were unaffected by the implantations and maintained their health and rate of growth unimpaired. The sacs were at first covered with a mantle of fibrin and wandering cells and later isolated by dense connective tissue capsules which closely confined their contents after the sacs had broken. No evidence was found of the invasion of other tissues by the micro-organisms, and the slight absorption of antigenic material from sacs and fibrous pockets was only sufficient to maintain a relatively low antibody titer in the blood serum.

The conditions produced bear some similarity to the typhoid carrier state and show experimentally the possibility of an indefinite parenteral survival of *Bacterium typhosum*.

REFERENCES

GATES, FREDERICK L. 1921 Jour. Exper. Med., **33**, 25.

MCCRAE, J. 1901 Jour. Exper. Med., **5**, 635.

ZINSSER, H., AND RAYMOND, E. H., JR. 1921 Proc. Soc. Exper. Biol. and Med., **43**, 121.

A NEW- OR DELTA-TYPE STREPTOCOCCUS

CARRIE KIRK BRYANT

From the Department of Microbiology and Bacteriopathology of the Thomas W. Evans Museum and Dental Institute School of Dentistry, University of Pennsylvania, Philadelphia

Received for publication June 27, 1924

INTRODUCTION

In making some routine studies of streptococci isolated from root canals of pulpless teeth at the Evans Institute, I have found a type which can not be classified as any one of the four outlined by Brown (1919).

The following description taken from Brown's work, will give the general characteristics of these four types.

Type α . . . . at the end of approximately 48 hours incubation . . . . To the eye the deep colonies appeared as small biconvex greenish colonies . . . . surrounded by zones . . . . in which the blood corpuscles were distinctly greenish in color. Under the low power of the microscope . . . . the greenish zone was less apparent, but in the outer portion of the zone there was possibly a slight hemolysis . . . .

After refrigeration for forty-eight hours . . . . Examined . . . under the low power of the microscope the deep colonies were seen to be surrounded by what may be called "double zones" . . . . composed of a distinct inner collection of . . . . greenish non-hemolyzed corpuscles next to the colony and an outer more or less hemolyzed ring . . . . Apparently the greenish discoloration of the corpuscles was accompanied by a fixation against hemolysis for (1) it preceded hemolysis, (2) the hemolytic substance must have passed out from the colony through the collection of corpuscles, and (3) it must have been present in greater concentration among these corpuscles next to the colony than farther out where hemolysis did occur. . . . As it has been noted that the zone of greenish corpuscles was formed during incubation and that hemolysis took place during refrigeration,

the blood agar plate was replaced into the incubator for another period of forty-eight hours. There appeared outside the hemolyzed zone another ring of greenish corpuscles . . . . Still another exposure to the temperature of the refrigerator resulted in the formation of a second ring of partial hemolysis . . . . Apparently the alternate throwing off of rings of fixed corpuscles and of hemolysis in response to alternate incubation and refrigeration is limited only by the exhaustion of the medium and the accumulation of metabolic products which inhibit further growth and activity.

Type β. This type of appearance in blood agar may be described as a colony surrounded by a perfectly clear, colorless zone of hemolysis. . . . . There is no trace of discoloration and when viewed microscopically no corpuscles are seen in the medium surrounding the colony. . . . . The colony itself is grayish by transmitted or reflected light . . . . after refrigeration for forty-eight hours they showed no change . . . . nor did they change during a second period of incubation and refrigeration. . . . . The difference in behavior of the two types in respect to refrigeration is so great as to suggest different hemolytic substances or processes.

Type α' . . . . It can scarcely be called anything but a hemolytic streptococcus for there are distinct hemolyzed zones and no apparent discoloration . . . . colonies of the alpha prime type appear somewhat hazy or poorly defined within the hemolyzed zones. Under the microscope . . . . the reason for this haziness is apparent; a few corpuscles are seen to remain throughout the zone but are most numerous next to the colony. . . . . After forty-eight hours incubation the plate . . . . was refrigerated for forty-eight hours. The zones were noticeably enlarged . . . . Placed into the incubator for another period of forty-eight hours the zones became still larger and rather diffuse but no multiple zones of concentric rings were formed.

Type γ. . . . . By the gamma type is meant the growth of the streptococcus colonies within and on the blood agar plate without the production of any perceptible hemolysis or discoloration of the surrounding medium during incubation or refrigeration.

ORIGINAL OBSERVATIONS

Grown in 5 per cent blood agar plates, using defibrinated horse blood, the surface colonies of the type under consideration produce no visible change in the surrounding medium. The deep

colonies after forty-eight hours incubation appear macroscopically, irregularly shaped, olive green in color and about 0.75 mm. in diameter. Each is surrounded by a pale green zone slightly narrower than the diameter of the colony. The ill-defined outer margin of this green zone is not surrounded by a clear colorless zone, but merges directly into the unchanged blood agar beyond. This appearance is unchanged by forty-eight

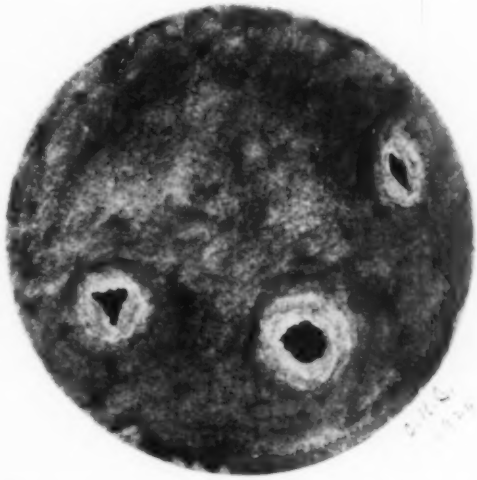


FIG. 1. PLATE COLONIES OF ORGANISM GROWN IN 5 PER CENT BLOOD AGAR, DRAWN AFTER FORTY-EIGHT HOURS INCUBATION AND FORTY-EIGHT HOURS IN ICE BOX SUBSEQUENTLY

There were approximately 175 colonies on the plate. Naked eye study seen by transmitted light and × ca. 8 diam.

hours subsequent subjection to refrigeration (fig. 1). Under the low power of the microscope in the zone immediately surrounding the colony the blood cells are seen to have lost all their pink color and to appear as minute pale grey-brown dots or when seen in clumps, as slightly darker grey-brown masses. There is no apparent decrease in their number, and they lie on an absolutely clear colorless back ground. The outer margin of this zone is very ill-defined and merges gradually into the unchanged medium where the agar is tinted a distinct pink from the laked hemoglobin

and the blood cells have retained their normal tone—the clumps especially being seen as definitely red masses (fig. 2).

The colonies resemble Brown's alpha type in that they are immediately surrounded by a zone, greenish to the naked eye, in which the blood cells still persist but without their normal color. But they differ from the alpha type in that refrigeration fails to produce an outer zone of hemolysis. They differ

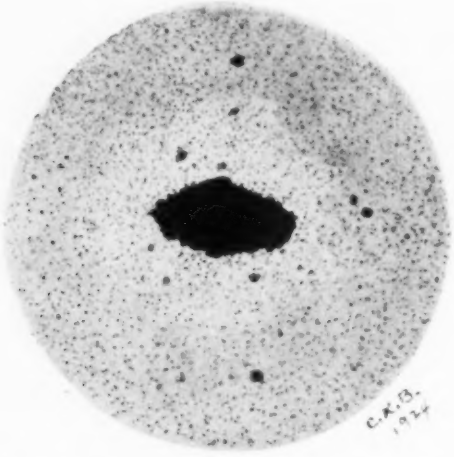


FIG. 2. COLONY TAKEN FROM FIG. 1 AS SEEN THROUGH LOW POWER OF THE MICROSCOPE, SHOWING COLONY AND ZONE IMMEDIATELY SURROUNDING IT, WITH SINGLE AND SMALL CLUMPS OF ERYTHROCYTES, GREY-BROWN IN COLOR, IN A CLEAR COLORLESS BACKGROUND

Beyond this and merging into it are to be seen the erythrocytes as they appear throughout the rest of the plate, single ones as brownish or red-brown, and clumps always red, lying in a pale pink background. × ca. 30 actual size of colony.

from both the alpha prime and the beta types in that there is no decrease in the number of blood cells in the zone surrounding the colony.

That the action of these delta-type streptococci on blood agar is characteristic, and is not due to some environmental peculiarity, is proven by the fact that they have been found present in plates showing typical colonies of the alpha type in mixed cul-

CHART 1

Number of organism	2	4	5b	12c
Patient from whom isolated	Mrs. M.	Murphy	Mrs. Drake	Murray
Date isolated	5/16/24	5/17/24	5/15/24	5/18/24
Date of blood plates	5/19/24	5/19/24	5/19/24	5/28/24
Date of sugar and milk inoculations	5/27/24	5/28/24	5/28/24	6/ 4/24
Bile solubility	−6/10/24	−6/10/24	Not tested	−6/17/24
Liquefaction of gelatin	−24 hours 37°C.	+24 hours 37°C.	Not tested	+24 hours 37°C.
* Action on litmus milk	a c r (½)	a c d (part)	a c r (¾)	a c d (½)
Mannitol	−	a faint	−	a faint
Lactose	a	a c	a	a c
Salicin	a	a c	−	a c
Raffinose	−	−	−	−
Inulin	−	−	−	−
Sucrose	−	−	−	−

* All milk and sugar records made at seventy-two hours. a = acid; c = coagulated; d = digested; r = reduction of litmus; − = negative result; + = positive result.

ture. When isolated and replated in pure culture they have retained their characteristic action in blood agar plates through repeated generations.

The morphological study was made from the water of condensation of an agar slant which had been incubated for twenty-four hours after inoculation with a typical deep colony picked from a blood plate. The surface growth on agar was frail, effused and filmy. The organism appeared as a Gram-positive micrococcus about 0.75 micron in diameter, in short chains varying from four to about fifteen elements and in irregular clumps.

By reference to chart 1 it will be seen that of the four strains isolated, all acidified and coagulated milk, all were bile insoluble, and none fermented inulin, raffinose, or sucrose. They varied in their proteolytic action on milk and gelatin.

We seem then, in these organisms, to be dealing with true streptococci which are of a type until now undescribed as to their behavior in blood agar plates.

REFERENCE

BROWN, JAMES HOWARD 1919 The use of blood agar for the study of streptococci. Monographs of The Rockefeller Institute for Medical Research, January 21, No. 9.

OLD STOCK CULTURES AS A SOURCE OF BACTERIOPHAGE

W. L. MALLMANN

Research Assistant in Bacteriology, Michigan Agricultural College

Received for publication May 23, 1924

Attempts to determine the nature of bacteriophage have been numerous but inconclusive. Two explanations have been advanced, the first by d'Herelle who claims that the bacteriophage is a living microorganism, parasitic upon bacteria; and the second by Bail and others who claim the lytic substance to be an autolytic product liberated by the decomposition of the bacterial cell. Considerable evidence in support of both theories can be found in the literature.

Bail (1921) reported the isolation of a lytic principle from three old broth cultures active against Flexner dysentery bacilli. Later Otto and Munter (1921) succeeded in 9 cases, in isolating a lytic principle active against members of the typhoid-dysentery group. These lytic principles were obtained from old broth cultures of dysentery and typhoid bacilli, the age of which varied from three weeks to six months.

Kuttner (1923) reported the isolation of a lytic principle from a bacteriophage resistant stock strain of *Bacillus typhosus* which was kept in flasks at 37°C. for four months and for two months at room temperature. Another lytic principle was isolated from a two-months-old culture of *Bacillus typhosus*. This was active only against Shiga dysentery. This author also reported that a co-worker obtained a lytic principle from young broth cultures of staphylococci.

Flu (1923) states that the bacteriophage is not a bacterial by-product, but a living parasitic organism.

The writer has attempted to examine a series of the old stock strains of laboratory cultures that had been grown on artificial

media for a number of years. Many of these cultures have been in the laboratory for ten to twelve or more years without any invigoration by animal passage or other means during that time. A number of non-pathogens were included in this study as it was felt that if the lytic principle was merely a bacterial decomposition product it should be produced by non-pathogenic bacteria as well as pathogenic.

The organisms were planted in extract broth, pH 8.0, and incubated at 25°C. Smears on liver agar plates were made at the end of twenty days to determine whether or not the cultures were still alive and also to study the colonies produced with the hope of finding colonies undergoing lysis. With the exception of *Micrococcus tetragenus*, all the cultures produced colonies. None of the colonies showed any signs of lysis.

Later at the end of seventy-eight days incubation, smears were again made on liver agar. This time a large number of the cultures had died. Those that were still viable did not produce any colonies suspected of containing a lytic principle.

At six months, practically all of the cultures were dead. If the lytic principle is an autolytic enzyme, it would seem that these dead cultures ought to show its presence. Accordingly these cultures were filtered separately, first through a filter paper previously impregnated with diatomaceous earth and then through a sterile Berkefeld filter. Tubes of sterile broth were next inoculated with the same stock culture from which the filtrate was originally prepared. The filtrate was now added to duplicate tubes in amounts of 1 drop, 2 drops and 2 cc. The cultures were incubated at 25°C. for twenty-four hours when readings were made. The cultures containing the filtrate were now filtered as before and again added to freshly inoculated tubes of the organism in question. This procedure was repeated four times. Four trials were made with the hope that any weak lytic principle that might be present would be invigorated and thus show its lytic action.

The following cultures were examined in this manner: *B. subtilis*, *B. megatherium*, Rector 24 Bacillus, *B. mesentericus*, *B. mycoides*, *B. ramosus*, *B. prodigiosus*, Smoke 29, Bacillus, *B.*

aerogenes, *B. violaceus*, *Ps. juglandis*, *B. cloacae*, *Ps. lucifera*, *B. carotovorus*, *Ps. pyocyanea*, *M. tetragenus*, *Msp. metchnikovi* and *Staph. aureus*. One trial was made with *Staph. aureus* and *Msp. metchnikovi*, three trials with *M. tetragenus* and four trials with each of the other cultures. All results were negative.

A culture of an old laboratory strain of *Bacillus typhosus* was incubated in broth at 37°C. for two weeks when an examination for lytic products was made. The filtrate from this broth culture was tested against *Bacillus typhosus*, *Bacterium dysenteriae* (Flexner) *Bacillus paratyphosus* A, *Bacillus paratyphosus* B and *Bacillus coli*. In all cases negative results were obtained. Plates smeared from all of the tubes showed only the usual normal colony.

To determine whether the medium used had any influence on the bacterial by-products produced, plain broth and peptone water were inoculated, using four different strains of *B. typhosus*. These cultures were incubated at 37°C. for thirty days when filtrates were prepared and tested as usual against all four different strains of *B. typhosus*. All results were again negative.

Cultures of *B. typhosus* and *Bact. dysenteriae* (Flexner) were incubated at 37°C. for two months and then examined for lytic principle, testing the filtrate against four different strains of the organism in each case. Negative results were obtained in all cases.

On the other hand, these same cultures of typhoid and dysentery used were always acted upon by bacteriophage obtained from feces and other sources.

The writer has obtained bacteriophage in one instance from a stock culture of *B. typhosus*, but this culture had been recently isolated and it is possible that the lytic principle was one of intestinal origin which had persisted in the culture.

REFERENCES

BAIL 1921 Weiner Klin. Woch., **34**, 447–449.
FLU 1923 Cent. für Bakt. Orig., **90**, 362.
KUTTNER 1923 Jour. Bact., **8**, 49.
OTTO AND MUNTER 1921 Deut. Med. Woch., **47**, No. 52.

THE BIOCHEMISTRY OF THE GRANULATED LACTIC ACID BACTERIA FROM CEREALS[1]

E. B. FRED, W. H. PETERSON AND H. R. STILES

From the Departments of Agricultural Bacteriology and Agricultural Chemistry, University of Wisconsin, Madison, Wisconsin

Received for publication July 1, 1924

The presence of granulated lactic acid bacteria in fermenting milk and in decoctions of cereal grains has been reported by various workers. These investigators have shown that the granulated bacteria represent a large group of organisms with certain well defined characteristics. All of the organisms of the group are gram positive rods, which grow best at temperatures above 37°C. and which form large amounts of active lactic acid in the destruction of the sugars. According to their occurrence, these organisms may be divided into two groups: first, the organisms commonly found in fermented milk which destroy lactose rapidly and produce high acidity in milk; second, the organisms found in fermenting mixtures rich in starch, which destroy lactose slowly and, after long incubation, may, or may not, curdle milk. It is this second group of the granulated rod forms found in fermenting starchy extracts of the cereal grain which is discussed in this paper.

Of the various kinds of microorganisms active in the fermentation of cereal decoctions, none are more striking in appearance or more quickly detected than the granulated lactic acid bacteria. These organisms, when stained with thionin or methylene blue, generally show the presence of distinct, round or oval-deep purplish-red bodies within the cell. Preparations from fermented milks (Düggeli, 1906; Hastings and Hammer, 1909) treated with methylene blue, thionin or Neisser's stain often

[1] This work was supported in part by a grant from the special research fund of the University of Wisconsin.

show the presence of rod forms with granules somewhat similar to those of the diphtheria bacillus. Photographs of these organisms from fermented milk are given in the reports of Kuntze, (1908) Heinemann and Hefferan, (1909) White and Avery (1910) and Orla-Jensen (1919); the plates in the last two papers are especially good. Under the name "Körnchenbacilli," in Yoghurt and Mazun, Luerssen and Kühn (1908) and Kuntze have discussed at length the characteristics of this group of lactic acid bacteria. From these papers it appears that the formation of granules is variable, depending on conditions, such as the kind of medium, the age of the culture, and the temperature. In a study of the rod forms of the lactic acid bacteria from Yoghurt, milk and cheese, Barthel (1912) also noted that granulation is not a definite characteristic. In general the granules are most noticeable during the period of greatest activity. On the other hand, White and Avery consider granulation a valid point of differentiation and in their study failed to note any loss or gain in this character. Lehmann and Neumann (1920 b) have suggested the name *Bacterium granulosum* for this group of the "Körnchenbacilli."

The presence of the so-called volutin granules or drops in bacteria from a totally different source, has been reported by Henneberg (1916) and by Thaysen (1921).

The former noted granules in the lactic acid bacteria of beer, the latter in starchy infusions of cereal grains. Thaysen gave this organism the name *Bacterium volutans* n. sp. *Fleming* and *Thaysen*, but this name seems hardly justified; since first, the description of this organism as given by Thaysen is far from complete; second, the occurrence of granules in lactic acid bacteria has been known for many years prior to the report of Thaysen, and third, granulation can hardly be called a fixed character but depends upon cultural conditions. In this connection the reports of Düggeli, Kuntze, Luerssen and Kühn, White and Avery, and Henneberg deserve special mention.

The organisms described by Thaysen are in cultural characters very similar to the bacteria studied by Henneberg, (1903, 1909), and by Steenberge (1920), and are no doubt closely related

to the lactic acid bacteria known as *Lactobacillus delbrücki* or *Lactobacillus cereale* (Orla-Jensen). From a review of the papers cited it is at once noted that the presence or absence of granules can scarcely be used as the main point to constitute a new and distinct species.

The organism described in the present paper would, according to the classification of the American Society of Bacteriologists, be designated as *Lactobacillus leichmanni* n. sp. (Henneberg).

The results here reported include a study of the structure of this organism, and of its general cultural characters as well as its qualitative and quantitative fermentation of various sugars and related compounds.

In order to become familiar with the general distribution of this group of lactic acid bacteria, a large number of cultures were isolated from various decoctions of fermenting cereals. The medium consisted of 0.25 per cent glucose in starch-free yeast water agar. On this substratum, the granulated bacteria develop rapidly, forming within twenty-four hours small pin pointed colonies which do not show any great increase in size on longer incubation. Cultures obtained in this way are rarely pure and should be replated several times.

From 50 pure cultures isolated from various sources, four were selected for detailed study. These were designated as cultures A, B, C, and D. Although these cultures exhibit slight differences in rate of growth, size of colony, amount of acid formed and degree of flocculation, it is thought that these variations are not great enough to warrant a division into separate species. The most important characters of this group follow.

GENERAL CHARACTERISTICS

Morphology. Size 0.6 to 0.8μ wide by 2.0 to 4.0μ long. Rods occur singly, in pairs, or in threads of three or more cells and frequently 50μ long. Rods may be straight or slightly curved with rounded ends. The long threads are frequently curled. The cells of young cultures in which the organisms are growing

rapidly are for the most part, single or in pairs. In old cultures there is usually a wide variation in form; sometimes very long and swollen threads are found.

Staining Reaction. Readily stained with the usual dyes. Young cultures, fifteen to twenty-four hours old, stain easily with thionin or methylene blue and show a distinct differentiation in the protoplasm of the cell. With Neisser's or Albert's

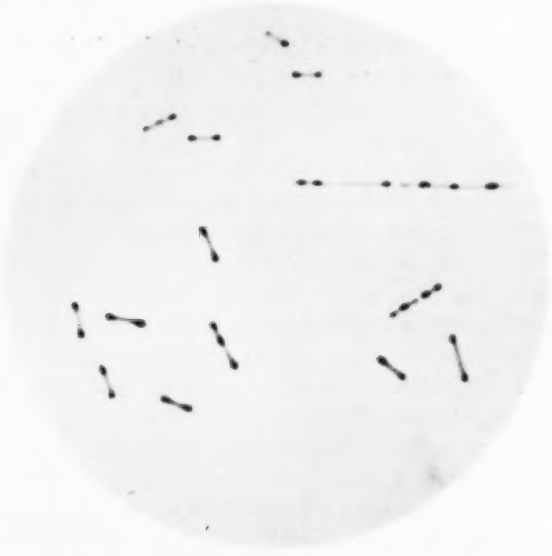


FIG. 1. CULTURE B. A TWENTY-FOUR-HOUR CULTURE IN GLUCOSE YEAST WATER. STAINED WITH DILUTE FUCHSIN AND DECOLORIZED. × 1200

stain, the granules are intensely colored. The cell wall appears blue and the granules a deep blue black or purplish red. The oval bodies or granules within the cell appear to be wider than the cell, and usually occur at each end of the rod. In some cases a third granule is found in the center. The shape and size of the granules depend to a great degree on the nutritive substance. This difference in the shape and size of the granules is clearly seen from figures 1 and 2. Although of the same age the granules in

the milk culture are decidedly different in shape and size from those in the glucose yeast water culture. The age as well as the kind of medium influence the formation of granules. Twenty-four hour corn mash cultures usually show long chains or threads which consist of many cells with granules. In the old cultures the granules are not so apparent.

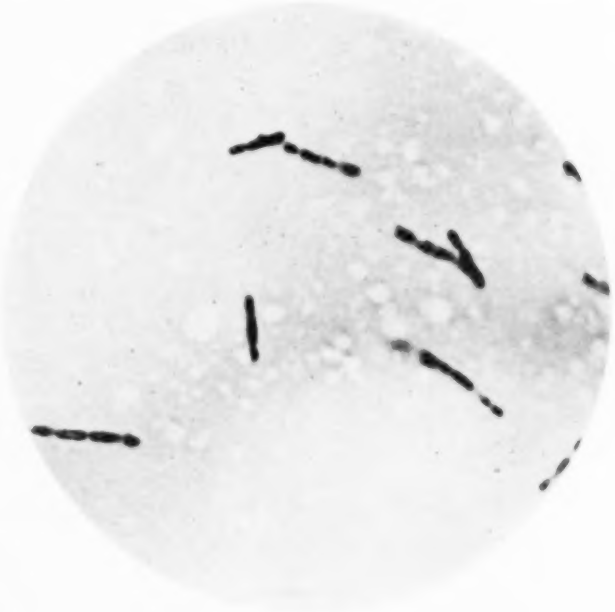


FIG. 2. CULTURE B. A TWENTY-FOUR-HOUR CULTURE IN MILK. STAINED WITH THIONIN. × 1200

Although it is beyond the scope of this paper to discuss the nature of these metachromatic bodies, it was noted that a suspension of this organism in water, heated to 80°C. for ten minutes fails to show granules. This loss of granulation or dissolving of the metachromatic granules in hot water agrees with the work of Meyer (1912). Mounts of twenty-four hour cultures, stained with thionin or fuchsin, showed beautiful granules before the heat treatment and afterwards no trace of granulation.

In unstained preparations prepared with nigrosin, the organisms stand out beautifully against a dark background. Mounts prepared in this way fail to show any granulations within the protoplasm.

Spores. None.

Capsules. None.

Flagella. None.

Gram stain. Positive.

Oxygen. Facultative anaerobic. Growth best under anaerobic conditions.

Glucose yeast water. No membrane, no gas, distinct clouding throughout and later a deposit.

Glucose yeast water agar stab. Growth along line of stab. Papillate.

Glucose beef peptone agar. A scanty growth along the stab. No surface growth.

Glucose yeast water agar colonies. Small pin point colonies which do not increase in size after longer incubation. The structure of the colony is similar to that of *B. anthracis* (Lehmann and Neumann, 1920a).

Glucose yeast water agar slants or streaks on Petri dish. A flat small whitish colony.

The surface colonies show under low magnification a mass of curled filaments projecting from the edge, usually in the form of parallel strands of hair-like lashes. Figures 3 and 4 are photographs of the margin of a twenty-four hour colony on glucose yeast water agar. Excellent photographs of this type of colony are shown in the report of Avery and White (1910).

The sub-surface colonies appear as minute wooly tufts with a margin of hair-like processes which radiate from the center. Although not confined to this one strain of lactic acid bacteria, the hair-like lashes from the margin of the surface colony are of some diagnostic value. Petri dish cultures after one or two days have a decided acid odor.

Glucose yeast water gelatin stabs. No liquefaction after six weeks incubation.

Potato. No growth.

Litmus milk. Acid and curdled in from ten to twenty days at 37°C. Curdling begins at the bottom of the tube first. Reduction of litmus after curdling. The curd is smooth, without gas bubbles or whey on top. After five weeks white granules are found on the surface of the curd.

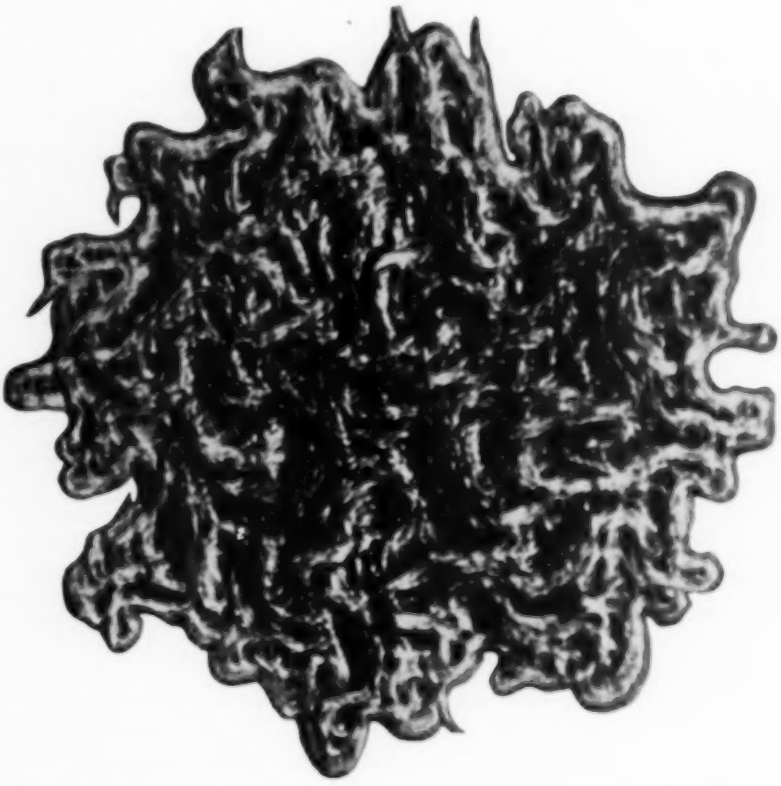


FIG. 3. CULTURE B. A TWENTY-FOUR-HOUR COLONY ON GLUCOSE BEEF PEPTONE AGAR. × 400

Reduction of methylene blue. Glucose yeast water medium containing (1 to 200,000) methylene blue is completely reduced in about five hours.

Glucose yeast water plus nitrate. Nitrate is not reduced.

Glucose yeast water plus sulphate. No reduction of sulphate.

Indol. Not formed.

Catalase. Negative.

Thermal death point. Not killed at 65°C. in ten minutes. Killed at 75°C. in ten minutes in a neutral medium.

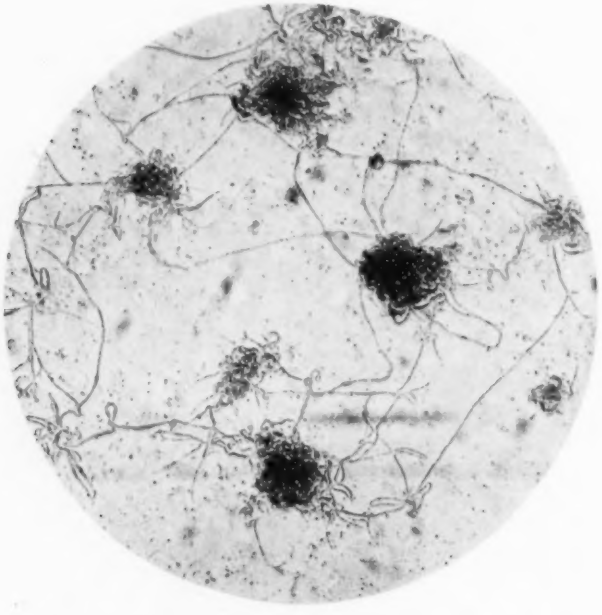


FIG. 4. CULTURE C. TWENTY-FOUR-HOUR COLONIES ON GLUCOSE YEAST WATER AGAR. × 50

Reaction. Optimum about pH 6.5. Growth range pH 4.0 to 8.0.

Acid formed. Mainly active lactic acid, which is laevo rotatory.

Pathogenicity. Nonpathogenic to rabbits.

Agglutination. Immune serum from rabbits agglutinates in dilutions of 1:100.

Temperature relation. Minimum 20°C. maximum 50°C. optimum 45°C. No growth after fifteen days at 12°C.

Comparison of growth at different temperatures. It has been pointed out by Henneberg, Orla-Jensen and others that the lactic acid bacteria from mash may be arranged in groups according to the temperature most favorable for growth. In an attempt to measure the temperature relation of these organisms two methods were used: (1) growth in agar stabs, (2) growth as shown by rate of acid production.

The results of the agar stab cultures are given below:

	AFTER 1 DAY	AFTER 2 DAYS	AFTER 5 DAYS
At 20°C	No growth	No growth	Poor
At 28°C	Fair	Good	Good
At 38°C	Good	Good	Good
At 45°C	Excellent	Excellent	Excellent
At 50°C	Fair	Good	Good

Growth, as measured by rate of acid production from glucose was the object of the next test. For this purpose cultures of all four strains were inoculated into 100 cc. portions of a 2 per cent glucose yeast water medium and kept at different temperatures. At varying intervals, a portion of the culture was removed with a sterilized pipette and titrated. The results of the acid formation from glucose are shown in the figures of table 1. A marked similarity was noted between the titration figures of the different strains, and hence only the data for culture B are given. Each figure of the table is representative of at least four parallel titrations. The results confirm those of former tests and show clearly that the high temperatures, 40°, 45°C. or above, favor the production of acids. On the other hand, low temperatures, 18° and 28°, are unfavorable and result in a very meager acid production.

FERMENTATION CHARACTERS

One per cent of the various carbon compounds in yeast water was tubed in portions of 10 cc. each. After sterilization, four

to six parallel tubes were inoculated with each strain of the lactic acid bacteria. The cultures were then incubated at 38°C. and at intervals of five, seven and ten days, the titratable acid and residual sugar (Shaffer and Hartmann 1920–1921) were determined. Repeated tests failed to show any increase in acid after seven days incubation.

According to the amount of acid formed in the absence of a neutralizing substance and the amount of sugar fermented, these substances may be arranged in two groups. The most readily fermented compounds are given first.

A. FERMENTED	B. NOT FERMENTED
Fructose	Arabinose
Glucose	Xylose
Mannose	Rhamnose
Maltose	Melebiose
Trehalose	Melezitose
Sucrose	Sorbitol
Salicin	Dulcitol
Galactose	Starch
Dextrin	Inulin
Lactose	
Manna flakes	
Raffinose	
Mannitol	
Glycerol	
Amygdalin	
α methyl glucoside	

Repeated tests show that it is difficult to draw a very definite and fixed line between these various groups. It is probable that some of the compounds placed in Group B, are attacked to a slight degree.

A much better idea of the fermentation of the more available compounds is shown in the figures of table 2. Here the total acidity and the percentage of sugar consumed are given. Without the presence of a neutralizing agent the lactic acid bacteria attack many of the sugars and related compounds vigorously, producing a marked acidity. Of the various compounds fermented, fructose, glucose, mannose, maltose and trehalose yield

TABLE 1

Effect of temperature on rate of acid production

0.1 N acid in 10 cc. of culture

TIME	CULTURE B				
	20°C.	28°C.	40°C.	45°C.	50°C.
hours	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
0					
8		0.2	0.5	1.1	0.9
16		0.3	1.9	3.0	2.3
24	0.1	0.8	3.3	4.3	3.5
32	0.1	1.0	4.0	4.8	3.7
40	0.2	1.1	4.7	4.9	3.8
56	0.5	1.4	5.3	5.2	3.8
72	0.6	1.4	5.3	5.2	3.8
168	0.7	1.5	5.4	5.8	4.5

TABLE 2

The fermentation of various sugars and related compounds

Incubated for seven days at 37°C.

CARBON COMPOUND	CULTURE A		CULTURE C		CULTURE D	
	0.1 N acid in 10 cc. culture	Sugar fermented	0.1 N acid in 10 cc. culture	Sugar fermented	0.1 N acid in 10 cc. culture	Sugar fermented
	cc.	*per cent*	*cc.*	*per cent*	*cc.*	*per cent*
Glucose	5.1	51	3.1	26	7.5	66
Fructose	5.5	56	5.2	44	7.4	65
Mannose	5.1	47	2.5	21	6.5	59
Galactose	4.2	31	2.0	13	2.0	15
Trehalose	4.1	33	3.5	19	7.4	49
Sucrose	3.6	23	3.3	26	6.8	49
Maltose	5.3	39	4.7	27	7.1	53
Lactose	3.3	10	1.7	3	1.8	Trace
Milk	1.8	Trace	0.5	5	0.5	2
Raffinose	2.2	17	2.4	15	3.6	26
Mannitol	1.6		1.8		2.9	
Dextrin	2.6		3.2		3.7	
Salicin	3.4		2.8		5.6	
Esculin	2.8		3.3		2.1	

the highest acid and consequently the highest percentage of sugar consumed. In spite of the fact that the organisms of this group are in the main acid formers, the figures of table 2 indicate clearly the advantage of combining the sugar analysis with the titratable acid measurement from the same culture. Titratable acid alone without additional determinations may lead to erroneous conclusions concerning the fermenting power of a given organism.

If the activity of the various cultures is compared, it will be seen that there are certain differences. Cultures A and B are quite alike and hence only the results for culture A are given. Culture D is uniformly more vigorous than the other strains in its attack on the carbon compounds except in the case of lactose. A possible explanation may be found in the difference in age of the cultures on artificial media. The two cultures A and B have been carried in the laboratory for almost two years while culture D was freshly isolated from acid wort. Culture C is different from the other three in that it makes a more abundant growth on solid media and does not ferment the sugars as vigorously as the other cultures.

For the diagnosis of these lactic acid bacteria, fructose, trehalose, maltose, salicin, dextrin, galactose and lactose are of special value. The pentose sugars are not attacked and of the four hexoses, galactose is the least fermented.

The quantitative data which follow give the main products of the fermentation.

THE FERMENTATION OF GLUCOSE, GALACTOSE, MANNOSE, FRUCTOSE AND MILK

Quantitative fermentations were carried out with cultures A and B. The results show that these cultures are true lactic acid producing organisms. Other products are insignificant. Alcohol, carbon dioxide, and volatile acid are found only in traces, hence the major reaction must be one in which the hexose molecule is cleaved into two equal parts. Lactose is attacked slowly, and is only partially destroyed in fifteen days. Glucose, fructose, galactose and trehalose are almost completely fermented.

It appears difficult for the organism to hydrolyze lactose. Trisaccharides show very little destruction, and their hydrolysis must present an even greater difficulty.

It was decided to limit the quantitative fermentations to glucose, fructose, galactose, and lactose since these sugars include all the monosaccharides obtained from the disaccharides and trisaccharides fermented by this organism. Quantitative organic analysis of bacterial cultures is always very difficult to adjust to an accurate basis. Minor discrepancies cannot well be eliminated. The type of fermentation flask and the methods of analysis were essentially those described in previous work (Fred and others, 1919, 1921). Extract of yeast containing about 80 mgm. of nitrogen per 100 cc. and adjusted to a pH of 6.8 previous to sterilization constituted the nitrogenous base in which the fermentation was conducted. An equal amount of yeast water, uninoculated, was analyzed for the control. These were subtracted from the corresponding figures of the fermented cultures. To avoid decomposition, the sugars were sterilized in 12 per cent water solutions and added to the yeast water. Carbon dioxide was absorbed in 30 per cent KOH. To neutralize the acidity sterilized 1 N NaOH was added from time to time in the presence of brom cresol purple or excess sterilized $CaCO_3$ was added.

Fermentation products. The weight of sugar fermented and the quantity of products formed are given in table 3. The data show practically complete fermentation of glucose and fructose and from 70 to 90 per cent destruction of lactose and galactose respectively. In the case of milk, one-half of the sugar was destroyed. Accumulation of end products of metabolism and a somewhat unsuitable sugar are probably the main factors which check the fermentation at this point.

The chief fermentation product is lactic acid. This acid represents from 90 to 95 per cent of the sugar destroyed. The quantity of volatile acid, probably acetic, is small; at most not more than 5 per cent of the sugar. The carbon dioxide produced by the organism is about 20 mgm. per gram of sugar destroyed and is evidently not a direct fermentation product but arises

from the respiration of the cells. Only traces of alcohol were found in the cultures; about 10 mgm. per 100 cc. for the monosaccharides and 35 mgm. for lactose. The above products account for approximately 95 per cent of the sugar fermented. Sodium hydroxide or calcium carbonate seem to be equally satisfactory as neutralizing agents.

TABLE 3

Total fermentation products from sugars

Calculated for 100 cc. of culture

CULTURE NUMBER	AGE OF CULTURE	SUGAR	ALKALI ADDED	SUGAR		VOLATILE ACID AS ACETIC	NON-VOLATILE AS LACTIC	CARBON DIOXIDE	TOTAL PRODUCTS	SUGAR FERMENTED
				Begin.	End					
	days			*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*
A	16	Glucose	NaOH	1.850	0.027	0.021	1.720	0.055	1.807	1.823
B	16	Glucose	NaOH	1.850	0.025	0.016	1.704	0.063	1.793	1.825
A	29	Glucose	$CaCO_3$	1.635	0.330	0.000	1.253		1.253	1.305
B	16	Fructose	NaOH	1.677	0.069	0.063	1.442	0.021	1.526	1.608
A	13	Fructose	$CaCO_3$	1.581	0.116	0.000	1.440		1.440	1.465
B	20	Galactose	$CaCO_3$	1.709	0.222	0.051	1.390		1.441	1.487
B	15	Lactose	NaOH	1.706	0.535	0.065	1.014	0.019	1.098	1.171
A	13	Lactose	$CaCO_3$	1.725	0.569	0.000	1.065		1.065	1.156
B	22	Lactose in skim milk	$CaCO_3$	4.420	2.167	0.000	1.917		1.917	2.253

TYPE OF LACTIC ACID PRODUCED AND WATER OF CRYSTALLIZATION OF ZINC LACTATES

The type of lactic acid produced is conveniently determined by making the zinc salt from the barium lactate and determining its water of crystallization. The active lactic acid salt crystallizes with two molecules of water whereas the inactive carries three molecules of water. Both salts are readily dehydrated at 105°. Table 4 gives the results of this work.

The results of the crystallizations show that this organism forms active lactic acid regardless of the kind of sugar. To determine whether the active lactic acid was dextro or laevo rotatory, 1.031 grams of anhydrous zinc lactate was dissolved in 25 cc. of water and the rotation observed through a 200 mm.

tube at 22°C. A reading of +1.76° on the Ventske scale was obtained which gives a specific rotation of +7.40. Hoppe-Seyler and Araki (1895) give +7.52 as the specific rotation of laevo zinc lactate. Since the rotation of the zinc salt is the opposite of the free acid it is evident that this culture of *L. leichmanni* produces laevo rotatory lactic acid.

TABLE 4

Water of crystallization of zinc lactates from various sugars

CULTURE NUMBER	SUGAR	CROP	WEIGHT OF SALT	LOSS ON HEATING AT 105°C.	WATER OF CRYSTALLIZATION*
			grams	*grams*	*per cent*
B	Glucose	1	0.4300	0.0552	12.84
B	Glucose	2	1.6704	0.2149	12.86
B	Glucose	3	0.3124	0.0402	12.87
A	Glucose	1	1.4771	0.1910	12.93
A	Glucose	2	0.9232	0.1182	12.80
B	Fructose	1	1.3973	0.1808	12.93
A	Fructose	1	1.0904	0.1420	13.02
B	Galactose	1	2.0286	0.2634	12.98
B	Lactose	1	1.3276	0.1716	12.92
A	Lactose	1	0.9504	0.1227	12.91
B	Lactose in skim milk	1	2.1112	0.2730	12.93
B	Lactose in skim milk	2	0.6738	0.0882	13.09
B	Lactose in skim milk	3	0.4306	0.0571	13.26

* Theory for $Zn(C_3H_5O_3)_2 + 2H_2O$ — 12.89 per cent water.

CONCLUSIONS

1. The granulated high acid forming bacteria of cereal infusions have been isolated and their cultural characters described.

2. These organsims are closely related to the high acid forming bacteria known as *Lactobacillus delbrücki*. Their cultural characters and fermentation reactions place them in the species described by Henneberg under the name *Lactobacillus leichmani*.

3. Glucose, fructose, and mannose are readily fermented. The fermentation of galactose is somewhat slower. Disaccharides are attacked to a lesser degree, and trisaccharides are scarcely consumed at all. Considerable acid is formed from glucosides and from dextrin.

4. Lactic acid is the major product of the fermentation. Only traces of volatile acid, alcohol, and carbon dioxide are found. The lactic acid produced is active and lalvo rotatory.

REFERENCES

BARTHEL, C. 1912 Zeitschr. f. Gärungsphysiol., **2**, 193–223.

DÜGGELI, M. 1906 Centralbl. Bakteriol., 2 Abt., **15**, 577–600.

FRED, E. B., PETERSON, W. H., AND DAVENPORT, A. 1919 Jour. Biol. Chem., **39**, 347.

FRED, E. B., PETERSON, W. H., AND ANDERSON, J. A. 1921 Jour. Biol. Chem., **48**, 385.

HASTINGS, E. G., AND HAMMER, B. W. 1909 Wis. Agric. Expt. Sta. Res. Bul. **6**, 197.

HEINEMANN, P. G., AND HEFFERAN, M. 1909 Jour. Inf. Dis., **6**, 304–318.

HENNEBERG, W. 1903 Centralbl. Bakteriol., 2 Abt. **15**, 154–170.

HENNEBERG, W. 1909 Gärungs bakteriologisches Praktikum, Betriebsuntersuchungen und Pilzkunde, Berlin, 443.

HENNEBERG, W. 1916 Centralbl. Bakteriol., 2 Abt., **45**, 50–62.

HOPPE-SEYLER, F., AND ARAKI, T. 1895 Jour. Physiol. Chem., **20**, 371.

KUNTZE, W. 1908 Centralbl. Bakteriol., 2 Abt., **21**, 737–768.

LEHMANN, K. B., AND NEUMANN, R. O. 1920a Atlas und Grundriss der Bakteriol., München, Teil I, Tab. 42, III and IV.

LEHMANN, K. B., AND NEUMANN, R. O. 1920b Atlas und Grundriss der Bakteriol., München, Teil II, 306.

LUERSSEN, A., AND KÜHN, M. 1908 Centralbl. Bakteriol, 2 Abt., **20**, 234–248.

MEYER, A. 1912 Die Zelle der Bakterien, Jena, 243.

ORLA-JENSEN, S. 1919 Mem. Aca. Roy. Sc. et Lettres, Denmark, **5**, 160–165.

SHAFFER, P. A., AND HARTMANN, A. F. 1920–21 J. Biol. Chem., **45**, 365.

THAYSEN, A. C. 1921 Jour. Inst. Brew., **27**, 529–542.

VAN STEENBERGE, P. 1920 Ann. de l'Inst. Pasteur, Paris, **34**, 803–870.

WHITE, B., AND AVERY, O. T. 1910 Centralbl. Bakteriol., 2 Abt., **25**, 161–178.

STUDIES ON TELLURIUM

THE VALUE OF SOME TELLURIUM COMPOUNDS AS DISINFECTANTS[1]

LOTTIE E. MUNN WITH B. S. HOPKINS

From the Chemical Laboratory, University of Illinois, Urbana, Illinois

Received for publication July 10, 1924

INTRODUCTION

In spite of the fact that the discovery of tellurium dates back to the latter part of the eighteenth century, no extensive practical use for the element or its compounds has as yet been found. Although it is properly classed as one of the rarer elements, comparatively large amounts of it could be recovered from flue dust and electrolytic slimes, if a demand existed.

The proximity of tellurium to sulfur, arsenic, and the halogens in the periodic table would suggest that its compounds might be poisonous and bactericidal in nature. Such has been found to be the case. The compounds which have been tested out so far as to their germicidal powers may be put in two general classes, i.e., the alkali salts of tellurous and telluric acids (Cavazutti, 1921; Lehmann, 1923; Joachimoglu and Hirose, 1920, 1921, 1922; Davis, 1914) and the tellurium derivatives of certain aliphatic B-diketones (Morgan, Drew with Cooper, 1922; Morgan, Cooper, Burtt, 1923). Tellurites are much more toxic than tellurates, and are especially active against bacteria of the colon-typhoid group. The resistance of an organism seems to be proportional to its power to reduce the tellurite to elementary tellurium, which is non-toxic to bacteria. The tellurium derivatives of the ali-

[1] Submitted by Lottie E. Munn to the Graduate School of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

phatic B-diketones are reported to have remarkable bactericidal and inhibitory powers.

The work which has been done indicates that tellurium compounds possess germicidal action, in some cases to a marked degree. The present investigation was undertaken with the purpose of still farther exploring this field and perhaps finding some compound of tellurium which would be equal or superior to some of our common disinfectants in efficiency without being prohibitive in cost.

DISCUSSION

It was considered desirable to try a compound containing tellurium as the positive part of the molecule, since no such salt had yet been investigated. Tellurium dioxide is more acidic than basic in its properties and, consequently, salts formed by the union of TeO_2 with acids, even such strong acids as nitric and hydrochloric, hydrolyze when the solutions are diluted, this process being hastened by the insolubility of tellurous acid as well as its weak ionization. For this reason, it was somewhat difficult to find a salt, in which tellurium played the part of the positive ion, which would be stable in the presence of water. Tellurium acid tartrate proved to be the most practical salt of this nature, as it was easy to make and showed no hydrolysis upon dilution. Since tellurium has a high atomic weight, 127.5, as compared with 107.88 for silver, it was thought that such a compound might have a bactericidal effect similar to silver citrate or lactate, both of which are used as disinfectants. Hageman (1919) gives an account of the preparation of this compound by prolonged heating of TeO_2 with a saturated solution of tartaric acid; this method was followed in the present investigation. Metallic tellurium was treated with nitric acid and the solution evaporated to dryness, the process being repeated until the metal had apparently all been converted to the dioxide, a white residue. In some cases this residue was a dirty white, indicating that some of the tellurium was not oxidized. In order to get pure tellurium dioxide, it was necessary to treat this residue with NaOH, filter, and precipitate the dioxide by means of an acid.

When dilute nitric acid was carefully added, the dioxide separated as a fine, white, crystalline precipitate, which settled rapidly; it was filtered off, washed repeatedly, and dried to constant weight. Assuming that the reaction would proceed as follows:

$$TeO_2 + 4H_2C_4H_4O_6 = Te(HC_4H_4O_6)_4 + 2H_2O$$

the amounts of the reacting substances, necessary to form 5 grams of the acid tartrate, were weighed out; to this mixture was added sufficient water to form a 5 per cent solution of the tartrate, and the whole was shaken without heating until the reaction was complete and a clear solution obtained; this solution showed no hydrolysis upon further dilution.

Although a very large number of organic tellurium compounds have been prepared, very few have been tried out as to their bactericidal effects; those which have been tested have been found to be efficient in this respect. In a recent article by Howard and Stimpert (1923) some experiments are described, which were carried on for the purpose of comparing the antiseptic action of aniline, zinc chloride, and the zinc chloride salt of aniline; the latter was found to exceed each of its constituents in disinfecting power. A glance at the formula of this compound $(C_6H_5NH_2)_2ZnCl_2$, at once suggested the idea that similar compounds of tellurium should have equal or greater bactericidal action, since tellurium salts are in general better germicides than zinc salts. Many tellurium compounds of this sort have been prepared (Lowy and Dunbrook, 1922). A definite amount of diethylamine was dissolved in dilute hydrochloric acid, and this solution was then added to a known excess of tellurium dioxide dissolved in hydrochloric acid, the latter being kept at sufficient concentration to prevent the separation of any TeO_2. Upon standing overnight, yellow crystals of diethyl ammonium tellurchloride, $[(C_2H_5)_2NH]_2H_2TeCl_6$ separated; these were filtered off, washed with ether, and dried. The aniline compound was prepared in an analogous way. The difficulty, which presented itself in the trial of these compounds as bactericides, was to find a suitable solvent. Water hydrolyzed them with the formation of tellurous acid; 95 per cent alcohol had the same effect; acetone dissolved

them but the subsequent addition of water caused hydrolysis. However, the aliphatic compound dissolved slowly in glycerol and this glycerol solution could be diluted with water, without the separation of tellurous acid. The stock solution contained 1 per cent of the diethyl ammonium tellurchloride, 30 per cent of glycerol, and 69 per cent of water. The aniline compound, on the other hand, showed very slight solubility in glycerol, and hydrolyzed upon addition of water, so it was not tried out as a disinfectant.

The wide use of silver salts as disinfectants and the marked bactericidal powers of the tellurites suggested the idea that perhaps silver tellurite would combine the properties of the two and exceed either in germicidal effects. In a rather comprehensive article on the metallic tellurites, Lenher and Wolesensky (1913) discuss the preparation of silver tellurite. In the present investigation it was prepared by adding the correct amount of silver nitrate to a solution of sodium tellurite, made by mixing a water suspension of TeO_2 with just enough NaOH to dissolve it. The silver tellurite separated as a pale yellow precipitate, which showed no change in color when dried and allowed to stand. Since, like all the heavy metal tellurites, it was insoluble in water, it was dissolved in ammonium hydroxide, forming silver ammonio-tellurite, and the latter was tried out as a disinfectant; the stock solution contained 1 per cent of the $[Ag\ (NH_3)_2]_2TeO_3$.

Iodine and its compounds have long been recognized as reliable disinfectants. Tellurium forms at least one well-defined iodide, TeI_4 (Hopkins, 1923). If potassium iodide is added to a solution of TeO_2 in hydrochloric acid, a black precipitate of tellurium tetraiodide forms immediately but dissolves upon further addition of potassium iodide to form a deep red solution of the double iodide, K_2TeI_6. A solution containing one part of the double iodide to thirty parts of solution was made up in this way. It showed no change on standing and could be diluted indefinitely.

Although the determination of phenol coefficients is not wholly satisfactory as a method for comparing disinfectants, it is the best means as yet devised for this purpose. The phenol

coefficient is obtained by dividing the figure indicating the degree of dilution of the disinfectant that kills an organism in a given time by that expressing the degree of dilution of phenol that kills the same organism in the same time under exactly similar conditions. The method used in the present work was analogous to the Hygienic Laboratory Method of Anderson and McClintic (Tanner, 1919).

In this investigation four test organisms were used, *Bacterium coli*, *Bacterium typhosum*, *Staphylococcus aureus*, and *Bacillus anthracis* (spores). In the case of the first three, twenty-four hour broth cultures were used, the culture having been carried over every twenty-four hours for three successive days using one 4-mm. loopful for the inoculation in each case. The anthrax cultures were grown for several days on agar slants until microscopical examination showed the presence of large numbers of spores. The growth was then washed off with physiological salt solution into a sterile flask and the vegetative cells killed by subjection to a temperature of 70°C. for fifteen minutes, leaving a suspension of spores only. By plating out dilutions of 1:100, 1:10,000, and 1:1,000,000 this suspension was standardized, and found to contain 30,000,000 spores per cubic centimeter.

By the use of sterile pipettes, sterile tubes, and sterile distilled water, the desired dilutions of the trial disinfectant solutions were made up, and exactly 5 cc. of each dilution placed in tubes to be later inoculated with the test culture. After the dilutions had all been made and the broth tubes for subcultures were in readiness, the broth culture of the particular organism was added to the disinfectant solutions with a sterile pipette in the proportion of 0.1 to 5 cc. of each dilution, and the tube was gently shaken several times. Plain broth subcultures were then made by means of a 4 mm. loopful from each dilution at the end of five, ten, fifteen, and twenty minutes. After incubation at 37°C. for forty-eight hours, the tubes were examined for growth as shown by cloudiness in the broth; in cases of doubt, agar plates were made from the tubes and examined for growth after incubation for forty-eight hours.

This method of determining phenol coefficients makes no

allowance for organic matter, the presence of which greatly reduces the efficiency in the case of some disinfectants. For this reason tests were carried out to ascertain to what extent the germicidal effect of the various test solutions was influenced by the presence of organic matter. It was arbitrarily decided to add 2.5 cc. of plain broth to 2.5 cc. of each dilution of the disinfectant, which was made double the desired strength to allow for the further dilution when an equal volume of organic matter was added. To this mixture of disinfectant and plain broth, which had a total volume of 5 cc., 0.1 cc. of a twenty-four-hour broth culture of *Bacterium coli* was added, and the tubes were then incubated at 37°C. for forty-eight hours, at the end of which time any growth was shown by the cloudiness of the broth.

RESULTS

The results of this investigation may be briefly summed up as follows:

1. The table below shows a comparison of phenol coefficients in the absence of organic matter.

	BACT. COLI	BACT. TYPHOSUM	STAPH. AUREUS
$Te(HC_4H_4O_6)_4$	2.50	6.25	0.18
$(C_2H_5)_2NH_2H_2TeCl_6$	6.12		
K_2TeI_6	212.50	218.75	363.63
$[Ag(NH_3)_2]_2TeO_3$	1000.00	9375.00	909.09
$AgNO_3$	625.00	11562.50	13636.36

2. In order to kill anthrax spores a twenty minute exposure to silver ammonio-tellurite, 1:300, to silver nitrate, 1:500, and to potassium iodotellurite, 1:40, was required.

3. In the presence of organic matter, the results indicate that silver ammonio-tellurite is effective against *Bacterium coli* in a dilution of 1:300,000, while silver nitrate ceases to be effective at 1:50,000. Potassium iodotellurite under the same conditions is greatly reduced in efficiency and must be present in a concentration of 1:2000 to kill *Bacterium coli*. Tellurium acid tartrate, however, seems to be effected but slightly by organic matter.

Although silver nitrate has a higher phenol coefficient than silver ammonio-tellurite against some organisms in water suspension, the fact that the latter is more effective in the presence of organic matter would tend to equalize this difference. The potassium iodotellurite is a promising compound, for wound and skin disinfection; it gives a yellow color to the skin, indicating the liberation of free iodine. Tellurium acid tartrate, while quite effective against *Bacterium coli* and *Bacterium typhosum*, seems to be rather weak against the coccus form; in order to kill the latter a 5 per cent solution would be necessary.

SUMMARY

1. As a disinfectant in the absence of organic matter, tellurium acid tartrate is more effective than phenol against *Bacterium coli* and *Bacterium typhosum*, but less effective against *Staphylococcus aureus*.

2. Diethyl ammonium tellurchloride is superior to phenol when used against *Bacterium coli* with no organic matter present; however, the ease with which it hydrolyzes reduces its practical value.

3. Potassium iodotellurite has a high phenol coefficient against *Bacterium coli*, *Bacterium typhosum*, and *Staphylococcus aureus*, in the absence of organic matter.

4. Silver ammonio-tellurite has a still higher phenol coefficient than potassium iodotellurite against the same organisms and under the same conditions; silver ammonio-tellurite has about the same disinfecting power as silver nitrate.

5. Against anthrax spores silver ammonio-tellurite is slightly less effective than silver nitrate; potassium iodotellurite is much less effective.

6. The disinfecting power of silver ammonio-tellurite is greater than that of silver nitrate in the presence of organic matter, using *Bacterium coli* as the test organism; potassium iodotellurite is greatly reduced in effectiveness by organic matter, while tellurium acid tartrate is not.

7. The potassium iodotellurite and the silver ammonio-tellurite seem to be most promising, the latter comparing favorably with silver nitrate as a disinfectant.

REFERENCES

CAVAZUTTI, A. 1921 Sul potere battericida dei soli di tellurio e di silenio. Ann. d'Ig., **31**, 551.

DAVIS, LEWIS 1914 A study of the tellurite reaction with the colon-typhoid group and other organisms. Centr. Bakt. Parasitenk., I. Abt., **75**, 180.

HAGEMAN, AARON M. 1919 A study of the preparation of certain organic salts of tellurium. Jour. Amer. Chem. Soc., **41**, 342–346.

HOPKINS, B. S. 1923 Chemistry of the Rarer Elements. D. C. Heath and Company, New York City, New York.

HOWARD, JOSEPH WHITNEY, AND STIMPERT, FRED O. 1923 The antiseptic action of the zinc chloride salt of aniline. Jour. Amer. Chem. Soc., **45**, 3106.

JOACHIMOGLU, GEORG, AND HIROSE, W. 1920 Die Wirkung ihrer Säuren auf Bakterien. Biochem. Z., **107**, 300; 1921. Die Wirkung ihrer Säuren auf Diphtheriebacillen. Biochem. Z., **125**, 1.

JOACHIMOGLU, GEORG 1922 Über die elektive Wirkung von Tellurverbindungen auf die Bacillén der Typhus-Coligruppe und ihre praktische Bedeutung für die Urologie. Z. Urol., **16**, 97–100.

LEHMANN, FRITZ 1923 Einwirkung ihrer Säuren auf Trypanosomen in vitro. Biochem., Z., **134**, 390.

LENHER, VICTOR, AND WOLESENSKY, EDWARD 1913 A study of the metallic tellurites. Jour. Amer. Chem. Soc., **35**, 718–733.

LOWY, ALEXANDER, AND DUNBROOK, RAYMOND F. 1922 Compounds of tellurium tetrabromide with organic bases. Jour. Amer. Chem. Soc., **44**, 614–617.

MORGAN, GILBERT T., COOPER, ENGLER A., AND BURTT, ARNOLD, W. 1923 The bactericidal action of tellurium derivatives of certain aliphatic B-diketones. Biochem. Jour., **17**, 30.

MORGAN, GILBERT T., DREW, HARRY DUGALD KEITH, AND COOPER, ENGLER A. 1922 Interaction of tellurium tetrachloride and B-diketones. Jour. Chem. Soc., **121**, 922.

TANNER, FRED W. 1919. Bacteriology and Micology of Foods. John Wiley and Sons, Inc., New York City, New York, pp. 148–160.

BIOLOGICAL INVESTIGATION OF PEAT

ARAO ITANO

From the Massachusetts Agricultural Experiment Station, Amherst, Massachusetts

Received for publication July 14, 1924

INTRODUCTION

Some general statistical material is offered in this paper[1] along with our biological studies of peat[2] because it establishes the pertinency of the work.

USES OF PEAT

The uses of peat, so far as known to the author, are shown diagrammatically in figure 1.

In the United States of America peat is used chiefly as a fertilizer. This is shown in table 1. Compare this table with table 2 which gives the production of peat.

DISTRIBUTION AND QUANTITY OF PEAT IN THE UNITED STATES

The distribution of peat deposit is given approximately on the map (1918 United States Geographical Survey, 11:15) in figure 2.

The amount of peat figured on air dried bases in the United

[1] L. J. Fitzpatrick and E. Tanner have assisted the author in some of the tests.

[2] The word, peat, as used throughout this paper is defined by A. P. Dachnowski, Bull. 802, Bureau of Plant Industry, U. S. Dept. of Agr., p. 10, December, 1919: "Peat is an accumulation of plant remains in various stages of disintegration or maceration, laid down in a definite manner according to imposed modifying field conditions. For statistical purposes and for reasons of common scientific interest the use of the term 'peat deposit' should be limited to an accumulation of at least 8 to 10 inches (20 to 25 cm.) in thickness when compact and well shrunk. In this accumulation the surface layer of living native vegetation or deposits containing more than 40 per cent of mineral matter must not be included."

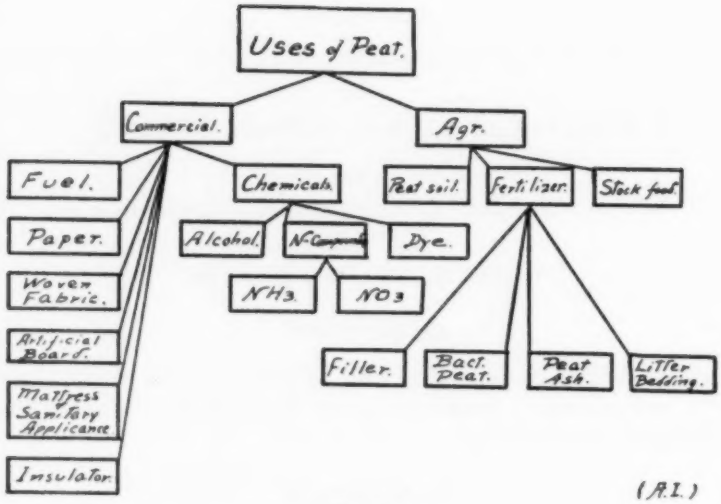


FIG. 1

TABLE 1*

Peat used in manufacturing fertilizer in the United States, 1915–1919

YEAR	QUANTITY (SHORT TONS)	VALUE	AVERAGE PRICE PER TON
1915	38,304	$258,447	$6.75
1916	48,106	336,004	6.98
1917	92,263	658,500	7.14
1918	79,573	775,313	9.74
1919	54,690	557,240	10.19

* U. S. Geological Survey, 11: 4, p. 41, 1919.

TABLE 2*

Peat produced in the United States, 1915–1919

YEAR	NUMBER OF PLANTS REPORTING	QUANTITY (SHORT TONS)	VALUE	AVERAGE PRICE PER TON
1915	9	42,284	$288,537	$6.82
1916	13	52,506	369,104	7.03
1917	18	97,363	709,900	7.29
1918	25	107,261	1,047,243	9.76
1919	15	69,197	705,532	10.20

*U. S. Geological Survey, 11:4 p. 41, 1919.

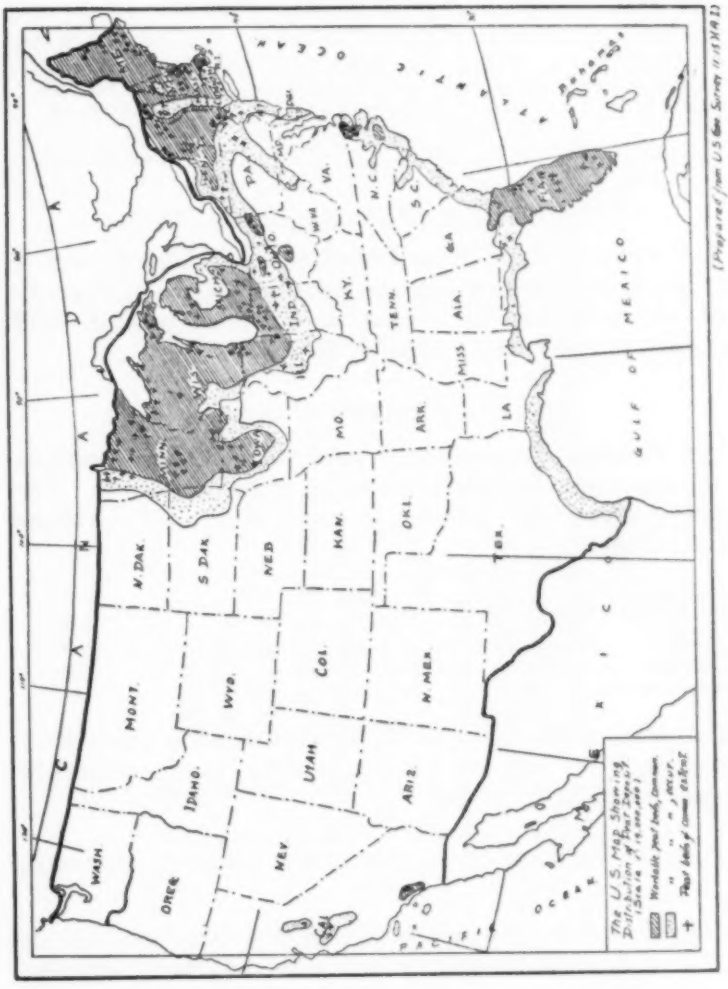

The U.S. Map Showing Distribution of Peat Deposits
Scale of 70,000,000
Workable peat beds common
" " " rare
Peat beds of some extent
(Prepared from U.S. Geo. Survey 11-1913)
CANADA
MEXICO
GULF OF MEXICO
ATLANTIC OCEAN
PACIFIC OCEAN
WASH
ORE.
CAL.
NEV.
IDAHO
MONT.
WYO.
UTAH
ARIZ.
N. MEX.
COL.
N. DAK.
S. DAK.
NEB.
KAN.
OKL.
TEX.
MO.
ARK.
LA.
MISS.
ALA.
GA.
FLA.
S.C.
N.C.
TENN.
KY.
VA.
W. VA.
PA.
N.Y.
ILL.
IND.
OHIO
MICH.
WIS.
MINN.

FIG. 2

States has been estimated by E. K. Soper[3] and others to be about 13,826,000,000 tons. It is distributed as follows:

	tons
Northern region	11,053,000,000
Atlantic coastal region	2,701,000,000
Other regions	72,000,000
Total	13,826,000,000

PRODUCTION OF PEAT IN THE UNITED STATES

The total production in the United States during the years of 1915 and 1919 inclusive is diagrammatically illustrated in figure 3.[4]

In Massachusetts, the output of air dried peat in 1918 amounted to 18,500 tons, valued at $160,200.[5]

CHEMICAL COMPOSITION OF PEAT

The chemical composition of peat varies widely according to the types and the conditions under which it is found. The general composition,[6] however, may be illustrated as follows (air dried bases):

Carbon	59.50
Hydrogen	5.50
Oxygen	33.00
Nitrogen	2.00
(Ash omitted)	

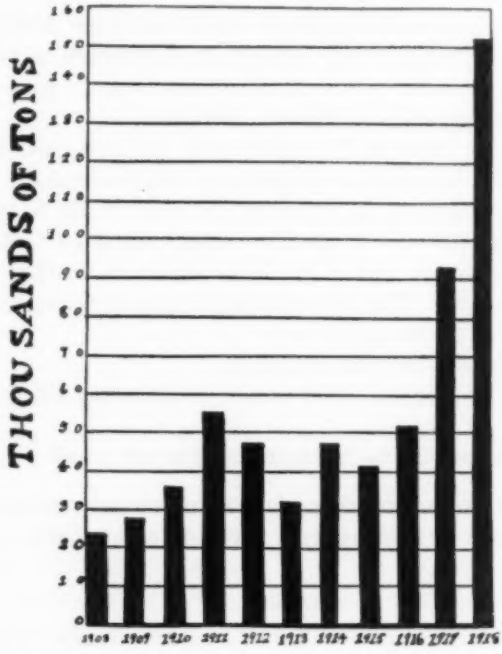
The peat which we used in our investigation came from Lexington, Mass. and was found to contain on an average 1.79 per cent nitrogen, dry weight.

UTILIZATION OF PEAT AS A FERTILIZER THROUGH MICROBIAL ACTION

The values of peat lie in its contribution to plant food and its physical action upon the soil. As a plant food its chief component

[3] U. S. Geological Survey, 11:15, p. 345, 1919.
[4] C. C. Osbon, U. S. Geological Survey, 11:15, p. 332, 1919.
[5] Ibid., 11:15, p. 337, 1919.
[6] C. C. Osbon, U. S. Geological Survey, 11:20, p. 264, 1917.

is nitrogen which exists in the amount of about two per cent of dry weight. Chemists have found that the nitrogen in peat consists of some complex organic nitrogen such as proteins, and other forms which are not very well understood. The amount of



Development of the domestic peat industry, 1908-1918.

FIG. 3

proteins[7] is about 26 per cent of the total nitrogen; of acid amides, 10 per cent; while the remaining portion is unknown.

Bacteriologists have prepared several kinds[8] of so-called "Bac-

[7] C. S. Robinson, Mich. Agr. Expt. Station, Technical Bull. 7, p. 22, 1911. The statement concerns only the sample investigated by the author.

[8] Bottomley and others.

terized" peat and they have claimed that this treatment increases the quantity of assimilable nitrogen. The degree of success in its application is subject to question in every case.

The method of investigation adopted in the present bacteriological study is as follows:

a. Isolation of microörganisms which produce the changes necessary for bringing about further decomposition of peat.

b. Addition of such types of microörganisms to peat for the purposes of instigating the changes desired.

c. Addition of foreign materials, such as barn-yard manure, which not only furnish microörganisms but food values as well.

In this investigation, an attempt has been made to alter the nitrogenous constituent of peat by supplying some factors and conditions which are seemingly lacking for its further decomposition in nature. The factors which have received particular attention in this investigation are:

a. The hydrogen-ion concentration.

b. The lack of essential food materials including vitamins.

PRESENCE OF MICROÖRGANISMS IN PEAT

The quantitative microbial determination was made on an air dried sample by plating with three different media as noted in table 3.

TABLE 3

Different culture media and microörganisms in peat

	NUTRIENT AGAR	ASHBY AGAR	PEAT EXTRACT AGAR
Number in 1 gram	1,500,000	5,140,000	1,200,000

Ashby's agar has given the greatest and peat extract agar, the smallest number of colonies.

The identification of the organisms which appeared as colonies on plates with the above media was executed according to the charts of the Society of American Bacteriologists. There were found ten species of bacteria, two of molds, some yeasts and torulae, and also protozoa (in the water culture).

SOME PHYSIOLOGICAL STUDIES OF THE BACTERIA ISOLATED FROM PEAT

Influence of H-ion concentration

The limiting reaction of the isolated species of bacteria was determined by the method described by Itano.[9] The results are given in table 4.

TABLE 4*

pH limits of the organisms found in peat

NUMBER OF BACTERIA	pH LIMITS
1	5.0– 9.0
2	5.0– 8.0
3	5.0– 9.0
4	5.0– 8.0
5	6.0– 9.0
6	4.0–10.0
7	5.0– 8.0
8	3.0– 8.0
9	5.0–10.0
10	5.0– 9.0

* The data were obtained by E. Tanner.

Since the hydrogen-ion concentration of peat itself lies between 1×10^{-3} to 1×10^{-4}, the growth and activity of these organisms are arrested. It is, however, true that many kinds of fungi tolerate such hydrogen-ion concentration; in fact, their growth on the peat is commonly observed.

Proteolytic action of the bacteria

The proteolytic action of these bacteria was determined approximately by the method described by Itano[10] and the results noted in table 5.

The results indicate that some of these organisms are as active in producing amino compounds as *B. subtilis* which has been previously investigated by the author.[11] The maximum

[9] Mass. Agr. College, Bull. 167, 1916.
[10] Loc. cit., p. 152.
[11] Loc. cit., p. 169.

proteolysis produced at the end of eight days was 142.80 mgm. of formol titrating nitrogen per 100 cc.

The foregoing determinations indicate the following facts:

a. The various microörganisms studied are present in peat.

b. The activities of these microörganisms are arrested by the concentration of hydrogen-ion and probably by the lack of some essential food elements as will be established later.

c. The larger number of the microörganisms present produce amino acids in considerable quantities.

TABLE 5

Proteolysis by the organisms found in peat

NUMBER OF BACTERIA	TOTAL FORMOL TITRATING N, IN MILLIGRAMS AFTER SEVEN DAYS (IN 100 CC. BROTH)
1	65.00
2	100.00
3	80.00
4	70.00
5	140.00
6	60.00
7	88.00
8	85.00
9	115.00
10	120.50

Change of nitrogen in peat by different treatments

Having demonstrated the presence of microörganisms in peat which are capable of disintegrating the nitrogenous substances and which also are ready to develop if the hydrogen-ion concentration of the peat is adjusted to their requirements, it will now be our aim to establish the influence of accessory food substances for the microörganisms as another important factor in releasing nitrogen from the more or less stable and non-usable compounds in peat and rendering it available for plant growth. For this purpose the following set of experiments was carried out, and the alkaline permanganate soluble and amino nitrogen was determined as indicated in table 6.

Where the initial reaction of the medium or material has been adjusted with NaOH and vitamin B and molasses added,

there has been found respectively 71.50 and 25.50 per cent of total nitrogen of the dried peat as measured by the alkaline permanganate soluble and the amino nitrogen (Van Slyke) methods.

TABLE 6

Change of nitrogen in peat by different treatments

NUMBER OF FLASKS	CONTROL AND TREATMENT	PER CENT OF TOTAL NITROGEN	
		Alkaline permanganate soluble nitrogen (A. O. A. C. method)	Amino acids (Van Slyke)
Control	1 gram of peat + 5 cc. H_2O	5.90	0.50
1	Control + m_8 NaOH to reaction pH 7.0	36.75	6.80
2	Control + m_8 Na_2HPO_4	33.50	5.70
3	Control + $CaCO_3$	32.00	4.60
4	Control + (1:1,000) vitamin B	33.50	5.80
5	Control + (1:100) molasses	43.00	11.00
6	Control + m_8 NaOH + (1:1,000) vitamin B	65.70	20.00
7	Control + m_8 NaOH + (1:100) molasses	55.00	18.50
8	Control + (1:1000) vitamin B + 1:100) molasses	45.50	12.50
9	Control + m_8 NaOH + (1:1000) vitamin B + (1:100) molasses	71.50	25.50

CONCLUSION

From these results it seems possible to render peat available as a nitrogen fertilizer when its hydrogen-ion concentration has been corrected and certain accessory foods have been added to stimulate the growth and activity of microörganisms already present.

THE OCCURRENCE OF A RED PIGMENT PRODUCING ORGANISM IN CORN MASH OF THE ACETONE BUTYL ALCOHOL FERMENTATION[1]

E. B. FRED, W. H. PETERSON AND W. R. CARROLL

From the Departments of Agricultural Bacteriology and Agricultural Chemistry, University of Wisconsin, Madison, Wisconsin

Received for publication August 18, 1924

The formation of a pink or red color on the surface of corn mash from plant cookers is not uncommon. This pigment is found not only in samples of corn mash cooked under plant conditions (240° to 280°F.) but also to a limited degree in mash prepared in the laboratory. A pale pink color is first noted on the surface of mash which has been allowed to stand for two to three days or longer and this pigment later changes to a dark red or reddish brown.

Since the pigment is not commonly found in the mash heated at low temperatures, but in samples heated above 100°C., it was thought at first that air contamination, possibly mold spores, might be the cause. No evidence, however, could be found to support this supposition. As a result of many tests it was found that samples of mash exposed to air contamination rarely developed a pink or red pigment. More conclusive evidence concerning the cause of the pigment was obtained from microscopic examinations of pink corn mash. Samples which were taken directly from the cookers at the end of the cooking cycle and from various parts of the filling line were stored at 37°C. Great care was taken to draw these samples under conditions which prevented outside contamination. At first it was thought that this contamination was due to molds; after

[1] This work was supported in part by a grant from the special research fund of the University of Wisconsin. The cultures were obtained through the courtesy of the Commercial Solvents Corporation of Terre Haute, Indiana.

two to six days, however, such mashes do not show the presence of mold threads but instead show numerous rod-shaped cells, with spores.

Presumably pigment formation is a product of the growth of a highly resistant spore-forming bacterium. In the present paper are reported the results of a study of bacteria found in this colored corn mash from the plant cookers. Some of the more important characteristics are described including the resistance of the spores to heat.

PROCEDURE

To obtain pure cultures of the causative organism, five samples of colored mash from different sources were diluted and isolation plates poured. Corn mash and glucose-beef-peptone-agars were used. Transfers were made from well isolated colonies on these two media and these sub-cultures were replated to insure purity. Without exception the types of colonies obtained from the various sources were alike in appearance, namely a thin spreading growth, of cream-colored colonies. The old colonies showed the presence of spores. Although white or cream-colored and in this respect unlike the growth in corn mash, the presence of spores suggested a possible relationship between these colonies and the chromogenic organism of the mash. When sub-cultures were made from these white colonies into tubes of corn mash and allowed to grow for two to three days, it was found that the colonies which were white on the agar medium produced a decided pink color in the corn mash. Because of this difference in color on various media, a series of tests was made in which different sources of carbon were tried. Of the various substances tested, none gave such a deep red pigment as potato or corn mash.

CULTURAL CHARACTERISTICS OF RED PIGMENT PRODUCER

Surface colonies, ten to twelve hours old consist of a group of wavy threads which radiate from the center. These threads become filamentous and continue to develop as the culture grows

older. Because of this peculiar filamentous growth, the young colonies of the red organism may be mistaken for the granulated lactic acid organism (Thaysen, 1921), a contaminant found in fermenting corn mash.

The rate of growth and general appearance of the bacterial mass is quite different from that of *B. mesentericus-vulgatus*. The results of many cultural tests showed that the general characteristics of this organism are almost identical with those described by Globig as early as 1888. It belongs to the Mesentericus group and has been described by Migula (1900), by Lawrence and Ford (1916), and also by Bergey as *Bacillus globigii* (1923), and by Lehmann and Neumann (1920), as *Bacillus mesentericus-ruber*. All the cultural and morphological tests as reported by these various investigators were carried out and with essentially the same results. It is, therefore, considered unnecessary to report all of these tests. There are, however, certain points which deserve special emphasis. Old potato cultures have a distinct odor of cooked ham.

Some of the cultural characteristics of *Bacillus globigii* (Migula) are given below:

Morphology. Occurs singly or in short chains. Towards Gram stain variable, some cells positive and some negative. Spores formed in old cultures after six to ten days. Actively motile in twenty-four-hour-cultures by means of peritrichous flagella.

Oxygen requirements. Aerobic.

Gelatin stab. Liquefaction. Shows a pale reddish brown color.

Broth. Fragile white scum.

Agar colonies. Growth spreading, filamentous, irregular, lobed, raised, cream colored.

Litmus milk. Slow peptonization and soft curd.

Temperature relation. Abundant growth at 37°C.

Catalase. Positive.

Glucose yeast agar slant. Thin, spreading growth over entire surface of medium. No pigment.

Potato agar. Thin spreading growth. A slight reddish brown color.

Corn meal agar. Similar to potato agar but very slight color.

Potato slope. At first a pink to red growth which rapidly spreads over the surface. After three days at 37°C., a dark reddish brown.

Corn mash. Surface after three days a faint pink and five to ten days later, a beautiful deep pink.

Starch. Hydrolized. After seventy-two hours at 28°C. a 1.5 cm. wide clear zone on starch agar plates.

Pigment production. *Bacillus globigii* grows rapidly over the surface of nutrient broth, and of agar, with or without various sugars, but fails to form any distinct red pigment. Approximately the same thing is true of cultures grown on media in which the beef-extract-peptone broth is replaced with yeast water. Towards the natural food products this member of the Mesentericus group behaves differently, producing on potato slopes and corn mash, a beautiful pink to red color.

The effect of varying the hydrogen ion concentration of the medium on the production of color was studied. It was found that within a wide range, change in reaction has only a slight effect on chromogenesis.

THE FERMENTATION OF VARIOUS CARBOHYDRATES

The fermentations were made as follows. One per cent of the desired compound, glucose, mannose, sucrose, fructose, maltose, lactose, and mannitol was dissolved in yeast water and this medium sterilized for thirty minutes at 10 pounds pressure. Duplicate flasks of each compound were inoculated with a young culture of *B. globigii* and incubated at 37°C. It was found that *B. globigii* attacks all of these carbon compounds. At first it forms a small amount of acid from these substances and this acid is later destroyed. If the culture medium is kept in shallow layers, the destruction of the sugars is rapid.

From the results of studies (Berthelot and Ossart, 1921; Fred, Peterson and Anderson, 1923) carried out with other members of the *B. mesentericus* group, for example, *B. vulgatus* it seemed probable that this red form would produce acetone and ethyl alcohol from the sugars. To test this point large cultures of glucose, xylose and corn mash were prepared. After sterilization these media were inoculated with spores of *B. globigii*, incubated at 37°C. for five days and analyzed. The methods for

measuring the products of fermentation have been described elsewhere (Fred, Peterson and Davenport, 1919; Fred, Peterson and Anderson 1921). Since no attempt was made to carry out the fermentation in a closed system, it is certain that the gaseous products and some of the volatile substances escaped. Within twelve hours after inoculation there was a profuse growth of *B. globigii* in all of the media.

In table 1 are given the results of the analyses of the glucose and xylose cultures. The corn mash failed to show any appreciable amount of acetone and ethyl alcohol and hence is not included in the table.

TABLE 1

Acetone and ethyl alcohol production from glucose and xylose

Calculated for 100 cc. of culture

	grams
Glucose:	
At beginning	1.86
At end	0.29
Fermented	1.57
Acetone	0.154
Ethyl alcohol	0.307
Xylose:	
At beginning	1.85
At end	0.79
Fermented	1.06
Acetone	0.029
Ethyl alcohol	0.051

The data are in agreement with an earlier report on aerobic pentose fermenters (Fred, Peterson and Anderson, 1923). Both glucose and xylose are rapidly fermented with the production of small amounts of acetone and ethyl alcohol. If calculated on the percentage of sugar consumed, the acetone equivalent for glucose is 9.8 per cent and for xylose 2.7 per cent. The alcohol is approximately twice as high, 19.5 per cent for glucose and 4.8 per cent for xylose.

CHANGES IN THE NITROGENOUS COMPOUNDS OF CORN MASH

Aside from the changes in the carbon compounds, the proteolytic action of *B. globigii* on corn mash was studied. The methods

outlined in a previous paper (Peterson, Fred and Domogalla) were followed. Unlike the acetone-butyl alcohol organism, it was found that the red pigment producer brings about only a slight hydrolysis of the proteins of the corn. After twenty-four days incubation the percentage of total nitrogen in the form of soluble compounds had risen from 13 to 24.1 per cent.

Resistance of B. globigii spores to heat. In the early paper of Globig great emphasis is laid on the heat resistance of the spores of this organism. He found that a temperature of 113° to 116°C. for twenty-five minutes is required to destroy the spores.

The object of the present test was to determine the heat resistance of *B. globigii* spores in a neutral medium and in corn mash. The method employed was similar to that employed by Bigelow and Esty (1920) and their co-workers. A suspension of spores, three weeks old on beef-peptone-agar was prepared. The spores were scraped from the surface of the agar and suspensions prepared in a neutral phosphate solution of pH 7.0. The total number of spores per cubic centimeter of this suspension was 250,000,000. One cubic centimeter portions were pipetted into special glass tubes 0.6 cm. in diameter and then sealed. These sealed tubes were subjected to a temperature of 115°C. for varying lengths of time. In this neutral phosphate solution it was found that the spores are alive after ten minutes at 115°C. but are destroyed after fifteen minutes.

A second test with four week old spores of *B. globigii* was carried out in a corn mash medium. The number of spores added to each tube was much smaller than in the first test, 2,530,000 instead of 250,000,000. The results follow:

Held at 115°C.

	TIME IN MINUTES									
	5	8	9	10	11	12	13	14	15	17
Germination of spores	+	+	+	+	+	+	−	−	−	−

In the corn mash medium the spores of *B. globigii* are killed after thirteen minutes. A similar test with a culture of 220,000,000 spores per cubic centimeter of *B. mesentericus-vulgatus* gave approximately the same results.

Because of the great heat resistance of *B. globigii* it is not surprising that it may be found in mash which has been cooked under pressure. No doubt the occurrence of this organism in the supposed sterilized mash depends to a large degree on the amount of contamination. Mash richly seeded with the spores of this organism is naturally much more difficult to sterilize than mash of the same concentration but low in numbers of spores.

THE ASSOCIATED GROWTH OF B. GLOBIGII AND B. GRANULOBACTER-PECTINOVORUM

The effect of various aerobic spore forming bacteria, *B. mesentericus-vulgatus*, *B. globigii* and members of the mesentericus

TABLE 2

Effect of Bacillus globigii on production of solvents by Bacillus granulobacter-pectinovorum in corn mash

NUMBER		YIELD OF SOLVENTS PER 1000 CC. OF CULTURE	AVERAGE
		grams	*grams*
1	*B. granulobacter-pectinovorum* alone	16.1	15.3
2	*B. granulobacter-pectinovorum* alone	15.9	
3	*B. granulobacter-pectinovorum* alone	14.3	
4	*B. granulobacter-pectinovorum* alone	14.8	
5	*B. granulobacter-pectinovorum* with *B. globigii*	14.7	15.2
6	*B. granulobacter-pectinovorum* with *B. globigii*	16.0	
7	*B. granulobacter-pectinovorum* with *B. globigii*	14.2	
8	*B. granulobacter-pectinovorum* with *B. globigii*	15.9	

group, on the growth of the acetone-butyl-alcohol organism has been studied. Since the mesentericus organisms are aerobic and the *B. granulobacter* anaerobic it is probable that their associated growth will show a symbiotic relationship.

The results of many tests indicate that this association is without injury to the butyl alcohol organism. Flasks of 5 per cent corn mash were divided into two groups. Five flasks received only the acetone butyl alcohol organism while five other flasks received the same organism and also *B. globigii*. After three days incubation at 37°C. the amount of solvents was determined.

The results of the analysis are shown in table 2. The results of all the experiments with the associated growth of these two organisms indicate that the two organisms may live together without any serious injury to the acetone-butyl alcohol production.

CONCLUSIONS

1. The pink or red pigment found on the surface of cooked corn mash may be caused by a spore former of the mesentericus group.

2. This organism is described in the literature under the name of *B. mesentericus-ruber* and *B. globigii*.

3. The spores of this organism are very resistant to heat. In a corn mash the thermal death point of the spores is 13 minutes at 115°C.

4. Color production is most noticeable on potato or on corn mash.

5. In a yeast water medium plus glucose or xylose, *B. globigii* grows rapidly and destroys a large part of these sugars. The non-gaseous neutral products of the fermentation are acetone and ethyl alcohol.

6. *B. globigii* may be grown in association with *B. granulobacter-pectinovorum* without any injury to the acetone butyl alcohol organism.

REFERENCES

BERGEY, D. H. 1923 Manual Determinative Bact., Baltimore, 300.

BERTHELOT, A., AND OSSART, E. 1921 Compt. Rend., **173**, 792.

BIGELOW, W. D., AND ESTY, J. R. 1920 Jour. Inf. Dis., **27**, 602–617.

FRED, E. B., PETERSON, W. H., AND ANDERSON, J. A. 1921 Jour. Biol. Chem., **48**, 385.

FRED, E. B., PETERSON, W. H., AND ANDERSON, J. A. 1923 Jour. Bact., **8**, 277.

FRED, E. B., PETERSON, W. H., AND DAVENPORT, A. 1919 Jour. Biol. Chem., **39**, 347.

GLOBIG. 1888 Zeitschr. f. Hyg., **3**, 323.

LAWRENCE, J. S., AND FORD, W. W. 1916 Jour. Bact., **1**, 297.

LEHMANN, K. B., AND NEUMANN, R. O. 1920 Atlas u. Grundriss der Bakteriologie, Mun., 472.

MIGULA, W. 1900 System der Bakterien, 554.

PETERSON, W. H., FRED, E. B., AND DOMOGALLA, B. 1924 J. Amer. Chem. Soc. **46**, 2086.

THAYSEN, A. C. 1921 Jour. Inst. Brew., 529–542.

INDOL AND SKATOL DETERMINATION IN BACTERIAL CULTURES

CARL R. FELLERS AND RAY W. CLOUGH

National Canners Association, Seattle, Washington

Received for publication August 11, 1924

The indol test has always been unsatisfactory from a bacteriological standpoint. The variability of the media employed, the diversity of methods, the inaccuracies of the tests and the dissimilar strains of the organisms used, all have tended to vitiate laboratory results. Various investigators have recognized the unreliable nature of indol tests in the characterization of bacterial species and have proposed not only many improvements to the original methods but have devised several new ones. The literature on the subject of indol and skatol detection and determination is voluminous and pertinent references, only, can be included in this paper. A brief summary of some of the more important contributions relating to the several methods most commonly used in bacteriological laboratories will, however, next be given.

THE EHRLICH ALDEHYDE REACTION FOR INDOL AND SKATOL

This test depends upon the production of a pink or red color with or without heat on the addition of para-dimethylaminobenzaldehyde to the acidified test solution. This red coloration is soluble in chloroform or amylic alcohol. The test was first proposed by Ehrlich in 1901. He showed that skatol gave under similar treatment a violet-blue color. Schmidt (1903) checked these color reactions and concluded that both indol and skatol could be accurately detected by the Ehrlich test. The test was applied to bacteriology soon after by Haenen (1905). Steensma (1906) found that upon the addition of dilute potassium nitrite the red color of indol was not changed, whereas the violet-

blue of skatol became deep blue and was soluble in chloroform. Deniges (1908) attempted to substitute cinnamic aldehyde and also vanillin for the more expensive Ehrlich reagent. This he did with varying success. He found that benzene, which he used to extract the indol, usually contained some substance which if present interfered with the color reaction. Boehme (1906) added potassium persulfate to the completed Ehrlich test for the purpose of intensifying the color. He also pointed out the application of the method to the study of bacteria. Baudisch (1915) called this improved Ehrlich-Boehme test very satisfactory for indol and skatol. Kligler (1914), Lewis (1915), and Crossonini (1910) all recommend the test as sensitive and accurate and more suitable for indol detection in bacterial cultures than the older Salkowski test with which they compared it. Weehuizen (1908) used the test in his investigation on the indol in flowers and found it gave very satisfactory results when applied to the distillate. He used potassium nitrite to intensify the colors. He found phloroglucinol gave tests identical with indol and that distillation was necessary to obtain accurate results. Blumenthal (1909) found that small amounts of skatol if present would be masked by the indol and vice versa, giving intermediate colors. He found the delicacy of the test to range from 1:1,000,000 to 1:5,000,000. He, as well as Homer (1915) suggested the use of spectroscopic methods in the identification of indol and skatol in the Ehrlich color tests. Herter (1905), also Herter and Foster (1905, 1906) determined indol quantitatively in mixtures by a combination of the Herter (beta-naphtholquinone sodiummonosulfonate) and Ehrlich tests using a double distillation process. Zoller (1920) rejected the Ehrlich method for indol in favor of the nitroso indol test performed on the distillate. His objections in the light of our present knowledge regarding the test cannot be considered valid. This investigator furnished a valuable contribution to our knowledge of indol determination by his work upon volatility, distillation and hydrogen ion concentration. The reaction of the Ehrlich reagent with indolacetic and indol propionic acids has been studied by Hopkins and Cole (1903), Herter (1908), and Salkowski (1919). A red color is formed

somewhat similar to the indol coloration though insoluble in chloroform. Among those who have used and recommended the test are Lewis (1915), Jordan (1919), Chen and Rettger (1920), Wenner and Rettger (1919), Van Loghem and Van Loghem-Pouv (1912), Zipfel (1912), Frieber (1921), Kolle and Wassermann (1913), Norton and Sawyer (1921), Barthel (1921), Cannon (1916), Horowitz (1916), and Rivas (1912),Goré (1921) and Levine and Linton (1924).

According to the Manual of Methods prepared by the Committee on Bacteriological Technic of the Society of American Bacteriologists (1923), the Ehrlich test is performed as follows:

> The reagent is a 2 per cent solution of paradimethylaminobenzaldehyde in 95 per cent alcohol. One cubic centimeter of this reagent is added to the culture, then drop by drop concentrated hydrochloric acid is added until a red zone appears between the alcohol and the peptone solution. Not more than 0.5 cc. of the acid is required. On standing the zone deepens and widens. The red color is soluble in chloroform and the test may be confirmed by shaking the culture with chloroform to see if the pigment dissolves. If it proves soluble the test is considered positive.

STEENSMA OR VANILLIN TEST FOR INDOL AND SKATOL

In 1906 Steensma proposed the substitution of vanillin for the more expensive para-dimethylaminobenzaldehyde which is used in the Ehrlich test. Weak indol solutions gave with a few drops of a 5 per cent alcoholic solution of vanillin an orange red color unchanged by sodium nitrite while skatol yielded a red violet color changed by this reagent to blue violet. The presence of skatol masks the red coloration due to indol. Deniges (1908) also made use of vanillin as well as cinnamic aldehyde to replace Ehrlich's reagent with satisfactory results. Weehuisen used this test in conjunction with Ehrlich's in his investigations on the indol in flowers. Blumenthal (1909) stated that while the vanillin and protocatechuic aldehyde tests were fairly satisfactory where indol alone was present, they proved inaccurate in mixtures of indol and skatol. Nelson (1916) made a thorough study of the Steensma vanillin test and compared the color reactions obtained

by the use of numerous alcohols, aldehydes and acids and the solubility of these pigments in various solvents. The nature of these color bodies is not known. Although he recommends hydrochloric or sulfuric acids, most of the acids are suitable for making these tests. Indol produces a deep orange color insoluble in chloroform while skatol gives a deep red to violet color soluble in chloroform. Although Nelson usually obtained very good results with this test, occasionally they varied greatly and were not satisfactory. Zoller (1920) scored the Steensma vanillin test and called it unreliable on account of the large number of interfering substances and the ease with which the colors become masked. Frieber (1921) indicated that this test gave positive results with indol, alpha-methyl indol and beta-methyl indol (skatol). The test is used in some laboratories to a considerable extent, principally to check the results obtained by other methods.

The committee on Bacteriological Technic of the Society of American Bacteriologists (1923) recommend the following procedure in carrying out the vanillin test for indol:

> To 5 cc. of the culture add 5 drops of a 5 per cent solution of vanillin in 95 per cent alcohol and 2 cc. of concentrated sulfuric acid. Indol gives a clear orange by this test which reaches its greatest depth in two or three minutes. Tryptophane on the other hand, gives a reddish violet which develops more slowly and deepens on standing or heating.

THE SALKOWSKI NITROSO-INDOL TEST (CHOLERA RED REACTION)

Salkowski (1883) proposed the use of concentrated sulfuric or nitric acid together with a very dilute solution of potassium nitrite. In the presence of indol a pink ring or coloration between the acid layer and the culture is formed. The color changes to blue green upon the addition of potassium hydroxide. Large amounts of indol produce a reddish brown precipitate. Where the culture already contains nitrites as in the case of *V. cholerae* the addition of nitrites is unnecessary. This was based upon the color reactions of nitrites formerly suggested by Baeyer (1870) and by Nencki (1875). Kitasato (1889) applied the test to bacteriology, while Nonotte and Demanche (1908) improved the

method of applying the test and attempted to make it quantitative. Zoller (1920) pointed out the necessity of distilling the substance which was being tested and indicated the inaccuracies of making indol tests directly upon the original cultures. He showed that skatol carbonic acid interfered with the test and added several refinements such as a direct distillation from a slightly alkaline solution and the application of the test to the distillate in a quantitative manner. He obtained very satisfactory results and considered the delicacy of the reaction to be about 1:500,000. In order to concentrate the color he extracted it with isopropyl or isoamyl alcohol. Tobey (1906) claimed that some slight differences are present in the color reactions obtained in the "Cholera red" and nitroso-indol tests. Baudisch (1915), Telle and Huber (1911), Crossonini (1910) and others have investigated this test but with the exception of Zoller and possibly Tobey most investigators have shown a preference for the Ehrlich test. Chen and Rettger (1920), Kligler (1913), Nelson (1916) Zipfel (1912), Wenner and Rettger (1919), Frieber (1921), and others have compared the Salkowski with the Ehrlich test for indol and all agree that the former is less delicate and less accurate than the latter. Frieber (1921) has demonstrated that indolacetic acid gives positive results with the Salkowski test, which is therefore not altogether reliable.

According to the Committee on Bacteriological Technic of the Society of American Bacteriologists in the Manual of Methods (1923), the Salkowski test is performed as follows:

Mix 5 cc. of the culture with about one-third its volume of 1:1 sulfuric acid. Then add on the surface a small amount of a 0.02 per cent solution of sodium nitrite. A positive reaction is indicated by a pink zone between the acidified culture fluid and the nitrite solution.

DIMETHYLANILIN TEST FOR SKATOL

To 5 cc. of the solution to be tested for skatol add a few drops of fresh dimethylanilin and shake vigorously. Add about 4 cc. of concentrated sulfuric acid to form a layer at the bottom. A violet ring is formed in dilutions of 1:1,000,000 or more. The color is soluble in chloroform. Indol does not interfere.

The United States Bureau of Chemistry in testing for the presence of skatol in decomposed foods also recommends this test. Clough (1922) modified the test by using hydrochloric acid in place of sulfuric acid and heating to bring out the pink color. The dimethylanilin should be recently redistilled else an interfering substance may obscure the color reaction. By this modified test a 1:5,000,000 dilution of skatol may easily be detected.

THE HERTER β-NAPHTHO-QUINONE SODIUMMONOSULFONATE TEST

Herter in 1905, and Herter and Foster (1905, 1906) proposed the use of β-naphtho-quinone sodiummonosulfonate for the detection and determination of indol. A double distillation is carried out first from the alkaline mixture and then on the acidified distillate. The addition of the reagent followed by potassium hydroxide in excess produces a blue condensation product of indol. The addition of acid changes this color to yellow. Now if this acid solution is redistilled, skatol if present, passes into the distillate and indol remains behind and may therefore be separated quantitatively. Gorter and DeGraeff (1908) claimed the method would give trustworthy results if a double distillation were carried out. They found ammonia interfered with the color reaction and that it must be eliminated before making the test. The delicacy is given as 1:8,000,000. Nelson (1916), also Zoller (1920) both compared this test with the other common indol tests but found that although it gave fairly dependable results, the reagent was almost impossible to obtain and that the test solutions of it deteriorated very rapidly. The test requires a long time to complete and they claim that ammonia and phenols interfere with the reaction. However Bergheim (1917) highly recommends the test particularly for the determination of indol and skatol in feces. A double distillation is necessary though the results are quantitative and skatol and ammonia do not interfere.

KONTO FORMALDEHYDE TEST

Konto in 1906 reported that formaldehyde added to the acidified distillate to be tested for indol yields a violet red color if

indol is present. Skatol under similar conditions gives a yellow or brown color. The delicacy is 1:600,000. Nelson (1916) also Clough (1922) did not find this test to be entirely trustworthy and therefore used other more suitable tests.

LEGAL NITROPRUSSIDE TEST

The Legal test (cited by Salkowski, 1883, also Hawk, 1918) for indol depends upon the formation of a blue color upon adding a few drops of a freshly prepared solution of sodium nitroprusside and making the resulting mixture alkaline with potassium hydroxide. If the mixture is now acidified with glacial acetic acid the violet color becomes blue. Deniges (1908) used the test with fair results and improved its technique somewhat by using an excess of acetic acid in the barely alkaline test mixture. The reaction is not as sensitive as the Ehrlich test. Frieber (1921) showed that indolacetic acid does not interfere with this test.

THE GNEZDA OXALIC ACID TEST

Gnezda (1899) found that oxalic acid, either in the solid form or in concentrated solution, takes on a red color with indol or with indol vapor. Morelli (1908), also Pittaluga (1908) applied the test to bacterial cultures. Blotting paper soaked in oxalic acid solution suspended over the test culture reacts very sensitively to indol. Zipfel (1912) found the test to give satisfactory results though he apparently prefers the Ehrlich test. Freund (1922), and also Baudet (1913), have reported satisfactory results with this test. Very recently Holman and Gonzales (1923) have fully recommended the oxalic acid reaction and claim it is reliable, simple, practical, and delicate. The action depends upon the volatility of indol and may be used in agar cultures as well as in liquid ones.

THE ESCALLON AND SICRE FURFURAL TEST

Escallon and Sicre (1906) recommended the use of furfural in testing for the presence of indol. The culture is first extracted with chloroform, the latter driven off by heat and the residue

taken up with a few drops of alcohol. The reagent is made by adding 1 gm. glucose to 55 cc. of hydrochloric acid, warming to boiling and making up to 100 cc. When the alcoholic residue is warmed with 3 cc. of the above furfural reagent, if indol is present a reddish orange color is produced.

THE DAKIN GLYOXYLIC ACID TEST

Dakin (1906) proposed the use of glyoxylic acid as a test for indol, skatol and tryptophan. To 1 cc. of the solution to be tested 1 cc. of the dilute glyoxylic acid solution is added and about 2.5 cc. of concentrated sulfuric acid. With indol a red to brown color forms at the zone of contact, while with skatol a deep red to violet coloration results. Homer (1914) studied the effect of formic acid, glyoxylic acid and glyoxal on the tryptophan derivatives and found that intensely colored substances were often formed in the presence of oxidizing agents.

THE CRISAFULLI PYRROL REACTION (PINE WOOD TEST)

Crisafulli (1895), Rohmann (1908), Hawk (1918) and others describe the following test for indol. Moisten a pine splinter with concentrated hydrochloric acid and insert it into the material under examination. If indol is present in appreciable quantity the wood becomes a cherry-red color.

SASAKI METHYL ALCOHOL TEST FOR SKATOL

Sasaki (1909) suggested the addition of 3 or 4 drops of methyl alcohol and an amount of concentrated sulfuric acid equal to the test solution. The acid must contain a trace of a ferric salt and the alcohol must be free from acetone. Skatol gives a reddish violet color whereas indol produces no color.

THE BAUDISCH OR NITROMETHANE TEST

Baudisch (1915) reported the following test for indol: A few drops of nitromethane followed by a little dilute potassium hydroxide are added to the test culture and heated. Cool and add one cubic centimeter of amyl alcohol, shake well and while

shaking add hydrochloric acid in excess. After the amyl alcohol has separated heat the mixture almost to boiling. In the presence of indol the amyl alcohol layer becomes red or rose-colored.

OTHER TESTS FOR INDOL AND SKATOL

Besides the several tests already described many others are mentioned in the literature on the subject. Most of them are really modifications of the aldehyde color reaction and make use of such substances as pyruvic aldehyde, glyceric acid and aldehyde, protocatechuic aldehyde and heliotropinpiperonal. Since most of them have no extensive use in the laboratory and are in general neither as sensitive nor as accurate as some of the better known and extensively used tests for indol and skatol, this discussion for the most part will be limited to the latter.

DISCUSSION OF THE TESTS

All the foregoing tests, with the exception of the glyoxylic acid of Dakin and the nitromethane test of Baudisch, were tried out by us experimentally in the laboratory using for most of the tests pure indol and skatol, as well as bacterial cultures and decomposed salmon. The Konto formaldehyde, Gnezda oxalic acid,[1] Escallon and Sicre furfural, pine wood, Sasaki methyl alcohol, pyruvic aldehyde and glyceric acid and aldehyde tests for indol or skatol proved to be unsatisfactory. These tests require fairly large amounts of indol or skatol to be at all reliable and even so, the color reactions were neither clear cut nor constant.

In the second list of tests, classed as fairly satisfactory though not entirely so, and for some reason or another not recommended for accurate work, are the Legal nitroprusside, Herter β-naphtho-quinone sodium monosulfonate, and the Salkowski nitrosoindol tests. Providing these tests are made on the distillate rather than on the culture direct, much better tests will be obtained. In the case of the Salkowski and Herter tests this pro-

[1] The excellent paper of Holman and Gonzales (1923) did not appear until after our laboratory work had been completed, hence in using this test we were not able to use the refinements suggested by these authors.

cedure is absolutely necessary on account of interfering substances such as indol-acetic acid, indol-propionic acid, ammonia, and alcohols and aldehydes, and other metabolic products which may be present in bacterial cultures. The indol acetic and indol propionic acids give a color reaction similar to indol, but since they are only very slightly volatile in water vapor, these products will be left behind. The Herter β-naphtho-quinone monosulfonate test for indol is useful in separating quantitatively indol from skatol, but the procedure is involved and the reagent exceedingly difficult to obtain.[2] The Salkowski test is not a delicate reaction and small amounts of indol may be entirely overlooked. A red coloration may indicate either indol, indol-acetic acid or indol-propionic acid, or all of them, if the test is made on the culture direct without distillation. Zoller (1920) has shown that the test is much more delicate and accurate when made on the distillate. He even obtained very satisfactory quantitative results. The nitroprusside test while accurate for considerable amounts of indol is not delicate enough for even ordinary routine indol tests.

The Ehrlich para dimethylaminobenzaldehyde reaction[3] was found to be by far the most accurate, reliable and simple test for indol or skatol when unmixed. When mixed together in a culture the color ranges from red to deep violet, according to the respective amounts present. For skatol alone the dimethylanilin test has been found to give very satisfactory results. In testing for skatol this should be made the primary test and the results should be checked by the Ehrlich paradimethylaminobenzaldehyde and Steensma vanillin tests. A dilution of skatol of 1:5,000,000 may be detected by the dimethylaniline test. It should be used on the distillate only.

Next to the Ehrlich test for indol and the dimethylanilin test

[2] We were unable to purchase this reagent in the U. S. Dr. O. Bergheim of the Jefferson College, Philadelphia, kindly furnished us with a liberal sample of this substance.

[3] The Gore (1921) modification of the Ehrlich test did not come to our attention until our study had been completed. Since this test depends upon the volatility of indol it should yield results comparable with the distillation test as described in this paper.

for skatol, the Steensma vanillin test for indol and skatol gave the most satisfactory results. It was found to be fully as delicate as the Ehrlich test though not as reliable. Interfering substances are occasionally present which mask or obscure the color reactions even when the tests are performed on the distillate. Experiments showed that hydrochloric acid could be substituted for sulfuric acid retaining at the same time the sensitiveness of the test but eliminating the charring effect of the concentrated sulfuric acid on the organic substances usually present. In an attempt to improve upon the use of vanillin as an indicator of indol and skatol a number of substituted vanillins were used. Methyl-vanillin, monobrom-vanillin and benzidine-mono-vanillin were tested out for both indol and skatol using both hydrochloric and sulfuric acids. It was found that these substituted vanillins proved less sensitive than vanillin itself. With skatol the violet blue product formed was entirely soluble in $CHCl_3$ while the orange product of indol was only very slightly soluble. In this way it is possible to effect a separation of indol and skatol. The following procedure gave very satisfactory results.

MODIFIED STEENSMA VANILLIN TEST

Make the test whenever possible upon the distillate (direct or steam). To 5 cc. of the solution being tested add 5 drops of 5 per cent solution of vanillin in 95 per cent alcohol, 2.5 cc. concentrated hydrochloric acid and mix. Indol gives a clear orange color, while skatol gives a violet to violet blue color soluble in chloroform. The orange color produced by indol is insoluble in this reagent. Delicacy for indol and skatol was found to be about 1:3,000,000. The application of heat hastens the reaction. Only definite positive results should be recorded.

THE MODIFIED EHRLICH PARADIMETHYLAMINOBENZALDEHYDE TEST

After much preliminary work using the various tests under a great variety of conditions, it was decided to make a detailed study of the Ehrlich reaction and if possible to eliminate errors, increase its delicacy, standardize the technic of the test and make it as nearly quantitative as possible.

It was found that discordant results were often due to varia-

tions in the color and thickness of the glass in the test tubes. Some of these tubes had a bluish green color which served to mask in part the faint pink color produced by small amounts of indol. Since it was attempted to record amounts of indol as small as 0.2 micromilligram of indol in the 5 cc. of test solution (1:25,000,000)

TABLE 1

Influence of the Ehrlich reagent on the color of the indol test

INDOL	EHRLICH REAGENT	COLOR PRODUCED
p.p.m.	*cc.*	
0.2	0.5*	Pure pink
0.2	0.75	Pink with slight yellow
0.2	1.0	Pronounced yellow
0.4	0.5*	Pure pink
0.4	0.75	Nearly pure pink
0.4	1.0	Pink with slight yellow
0.4	1.25	Pink with more yellow
0.4	1.5	Pink with still more yellow
0.4	1.75	Pronounced yellow
0.4	2.00	Pronounced yellow
0.8	0.5*	Pure pink
0.8	0.75	Deeper pure pink
0.8	1.0	Pink with trace of yellow
0.8	1.25	Pink with slight yellow
0.8	1.5	Pink with considerable yellow
0.8	1.75	Pink with strong yellow
0.8	2.0	Yellowish pink
2.0	0.5,* 0.75, 1.0	Pure pink
2.0	1.25	Pink with trace of yellow
2.0	1.5	Pink with slight yellow
2.0	2.0	Pink with considerable yellow

* Five-tenths cubic centimeter of the Ehrlich reagent was the amount selected as the optimum for small amounts of indol.

it was absolutely necessary to use tubes of uniform bore, thickness and color. Thin walled, colorless, uniform tubes were used in all these tests.

Paradimethylaminobenzaldehyde dissolved in alcohol has a yellow color; this, also, was found to interfere with the tests for indol and skatol, particularly when the latter are present in very

small amounts. Different amounts of indol in 5 cc. of water were treated with varying amounts of the reagent and the colors produced were noted. Ten drops of concentrated hydrochloric acid were used in each test, the color being extracted with chloroform.

Table 1 shows that as the amount of the Ehrlich reagent is increased in the test, the greater is the interference of the yellow color, this being particularly true where dilute concentrations are present. From these data and from other tests conducted at this time it was concluded that 0.5 cc. of the reagent (paradimethylaminobenzaldehyde) represented the optimum amount for small amounts of indol, 2.0 micromilligrams (0.4 p.p.m.) or less.

It was found that concentrated hydrochloric acid partially destroys the interfering yellow color of the Ehrlich reagent, though the ten drops of the former usually used in the test, has little effect. Amounts of concentrated hydrochloric acid varying from 0.5 to 2 cc. were used in experimental tests. The result was that as the acid concentration increased the percentage of color destroyed increased also. However a point was reached at 2 cc. hydrochloric acid where the usual pink color of the completed test changed very quickly to orange. This destruction of the pink color takes place especially rapidly if the tubes are heated. Since the orange color is objectionable, it was decided, considering all the factors entering into the test, that 1 cc. concentrated hydrochloric acid gave optimum results because at this concentration of acid a considerable percentage of the objectionable yellow color of the reagent was destroyed without affecting the final reading of the test.

Several experiments were carried out to ascertain the effect of heating on the indol test as it had been found that the color developed much more rapidly when heat was applied. However, the time of heating was found to have a decided influence upon the final test. It appeared that the hydrochloric acid partially destroyed the pink indol color if the tubes were heated for more than thirty seconds. After repeating several times an experiment to determine just how much heating should be given, it was found that an exposure of twenty seconds in boiling water

gave optimum intensity of color without any harmful effect of the hydrochloric acid being apparent. The color which developed in 20 seconds in boiling water was approximately equal in intensity to that produced after standing for twenty to twenty-five minutes at room temperature. It is therefore a saving of time to apply heat to the test tubes before making the final reading. In this connection it should be stated that before adding chloroform to extract the color, it is necessary that the test solution be below the boiling point of chloroform, e.g., 61.2°C.

DISTILLATION OF INDOL FROM CULTURES AND FROM AQUEOUS SOLUTIONS

Indol, skatol and some other products of decomposition are volatile with steam. In this investigation it was necessary to determine the percentage recovery of indol in the distillate. Zoller (1920) determined that a slightly alkaline reaction in the culture was best (between pH 8 and 10.5). He used a single direct distillation and found that nearly all the recoverable indol was recovered in the first 75 cc. distilled, the total volume being 100 cc. Several investigators have shown that only a part of the indol added to a solution or solid suspension is recovered by either direct or steam distillation.

In order to determine the effect of varying hydrogen ion concentrations upon the recovery of indol in the distillate, varying amounts (5 to 50 cc. of a 10 per cent solution) of both sodium hydroxide and hydrochloric acid were added to the regular distillation flasks in addition to 12 micromilligrams of pure indol and 500 cc. of water. The material was then steam distilled, 500 cc. of the distillate being collected and tested for indol. The average per cent recovery of indol of the nine distillations where the alkali was used was 46.4 per cent, the amount of alkali making little difference in the results. On the other hand the average per cent recovery of the five acid distillations was only 26.7 per cent. In none of these cases was the recovery very satisfactory yet the alkaline solutions yielded nearly twice as much indol in the distillate as the acid solutions.

Reducing the volume of the solution distilled for indol appeared

to increase the percentage recovery. In a series of distillations where the residual volume in the flask was varied from 310 to 65 cc. the percentage recovery increased from 26.6 to 53.3 per cent.

Little difference was noted as to whether steam or direct distillation was the more satisfactory. Since the concentration could be controlled and little bumping or frothing occurred, steam distillation was used in this work. Another reason for using steam distillation is because scorching of proteins forms either indol or an indol-like substance which gives some of the color reactions of indol. As a result of a large number of tests it was determined that the length of time of the distillation had little or no bearing upon the percentage of indol recovered in the distillate. When known amounts of indol were added to perfectly fresh, chopped up salmon in a flask with 500 cc. of water and distilled with steam, the recovery of indol varied from 47 to 65 per cent.

TABLE 2

Recovery of indol from bacterial cultures by steam distillation

DISTILLATE		TEST SOLUTION (5 cc.) DILUTED TO*	TOTAL AMOUNT OF INDOL
Number	Cubic centimeters	cc.	p.p.m.
1	100	40	2.56
2	100	10	0.12
3	100	10	0.02
4	100	5	0.01

* It was necessary to dilute the test solution to this volume because the red color due to indol was too intense for accurate comparison with standards.

In order to determine the percentage recovery of indol in the first 100 cc. of the distillate, a culture tube containing 10 cc. of Dunham's peptone solution inoculated with an organism from pink salmon which was known to produce indol, was incubated for one week and then distilled with steam. The distillate was collected in four 100 cc. portions and the amount of indol in each portion determined separately. The results are given in table 2.

These data indicate that it is unnecessary to distil more than 100 cc. when a 10 cc. culture is used for the test. Ninety-four

per cent of the total indol recovered was obtained in the first 100 cc. of distillate.

Another series of six experiments to determine the volatility of indol with steam from protein substances was performed. Decomposed salmon, known to contain considerable amounts of indol was used. Two hundred grams of the ground up flesh macerated in 200 cc. of water were steam distilled and the distillate collected in 100 cc. portions until about 10 such portions had been collected. The indol was then extracted with ethyl ether and estimated colorimetrically according to the modified Ehrlich technic. The average per cent of indol recovered in the first 500 cc. distilled was 82.7. The per cent collected in the first 100 cc. portion of the distillate collected was only 23.8. The indol in flesh appears therefore to be rather firmly held and it is only with difficulty entirely removed. Such a condition is analagous to the determination of ammonia in water and in certain organic substances. However, it is possible by using a fixed arbitrary technic, to obtain fairly comparable results. The Ehrlich test gives an accurate indication of the amount of indol or skatol extracted from the distillate; since, however only 80 or 90 per cent of the total indol is actually recovered the results obtained by the method are not absolute. Furthermore, it is impracticable to recover all the indol present in a culture or other substance and it is believed that the distillation of a certain amount of the material being tested and the collection of a definite amount of distillate offers the best method possible for the quantitative determination of indol and skatol in cultures or other substances. In the case of peptone, tryptophan or trypsinized protein solutions, the indol and skatol are more readily liberated than from the complex proteins such as are present in flesh or plants, and for this reason a higher per cent recovery might be expected where culture media contain these relatively simple substances.

Still another experiment to determine the recovery of indol in the distillate was carried out using Limburger cheese, a substance which contains much indol. One hundred grams of cheese with 100 cc. of water were distilled with steam and three, five

hundred cc. portions of distillate were collected. Each portion was extracted two or three times with ethyl ether and the indol present in each separate extraction determined. The results are given in table 3. This table further confirms previous experiments where fish flesh and culture solutions were used. Although the greater part of the indol is found in the first 500 cc. of the distillate, considerable amounts are also found in the second and third 500 cc. portions.

TABLE 3

Recovery of indol from Limburger cheese by steam distillation

DISTILLATION NUMBER	SUCCESSIVE EXTRACTION	TEST SOLUTION (1 cc.) DILUTED TO*	TOTAL AMOUNT OF INDOL
		cc.	*p.p.m.*
1	A	300	180.0
	B	20	16.0
	C	2	1.6
2	A	300	90.0
	B	20	6.0
3	A	30	48.0
	B	5	6.0

* The intensity of the red color due to indol made it necessary to dilute the test solution in order to accurately compare the color with that of the standards.

EXTRACTION OF INDOL FROM THE DISTILLATE

Referring to table 3, it is evident that one ether extraction in a separatory funnel serves to remove nine-tenths or more of the indol present in the distillate. This was found to be true regardless of whether the indol was distilled from flesh, bacterial cultures, or cheese. Petroleum ether has been recommended for the extraction of indol but in this investigation it was found to be inferior to ethyl ether. The petroleum ether contains impurities unless well purified, which seriously interfere with the indol color reactions, particularly in the vanillin test.

Upon vigorously shaking the distillate containing indol with ethyl ether, a persistent emulsion often resulted making it difficult to separate the ether layer from the aqueous layer. It was found

that c.p. HCl dispersed the emulsion and simplified the separation of the layers. Commercial acid is not satisfactory because of impurities which interfere with a clear color test. The addition of two cubic centimeters of the c.p. hydrochloric acid was found to give very satisfactory results in dispersing the emulsion while not making it too difficult to neutralize subsequently with sodium hydroxide. Although only approximately 50 per cent of the ether used in the first extraction separates from the water, practically all of the recoverable indol is obtained in the first extraction.

During bacterial decomposition substances other than indol and skatol which are volatile with steam, may interfere with the color tests for indol and skatol. Since phenols and cresols and certain other substances which might be present in a decomposing mixture, are soluble in dilute alkali, the acidified ether extract of the distillate was washed with 5 cc. of 2.5 per cent NaOH to remove such substances. Of course it was then necessary to rewash the ether extract with 5 cc. of dilute HCl to remove the alkali. Repeated experiments showed that unless this was done the color reactions were sometimes obscured.

EVAPORATION OF THE ETHER EXTRACT

After the ether extractions have been washed with alkali and acid they must be evaporated over a small volume of water. Usually 10 cc. is used unless it is desired to apply several different tests for indol and skatol. Ordinarily where the Ehrlich test is used for indol and the dimethylanilin test for skatol, 10 cc. of test solution is sufficient. It was found that there was always some loss of indol upon evaporation, regardless of the method used. Spontaneous evaporation was compared with both hot water immersion and water bath with aspiration. Some difference was noted in the several methods and for the sake of speed, the application of heat with aspiration appears desirable. Needless to say the same technic should be employed in every test. For the sake of uniformity, heating on the water bath until most of the ether has been evaporated and removing the last traces by aspiration is recommended.

COLOR TESTS ON THE WATER TEST SOLUTION

After the ether has been entirely removed from the flasks containing the test solutions, these are ready to be subdivided and the actual color tests made. Two 5 cc. portions are placed in thin walled, colorless glass test tubes; one of these is used for the test and the other held in reserve. The necessary reagents are added from burettes, the tube placed in boiling water for twenty seconds, plunged into ice or cold water until cool and the chloroform is then added to extract the color due to the indol or skatol. After thorough shaking the tubes are allowed to stand at room temperature until compared with the standards. Unnecessary delay at this point should be avoided as the color changes somewhat and decreases in intensity after some hours. The standards are prepared from standard indol and skatol solutions prepared in exactly the same way. Such solutions were conveniently prepared from stock alcoholic solutions containing 80 mgm. indol or skatol per 100 cc. These stock solutions when tightly stoppered retained their full strength for several months at least. On the other hand a word of caution is necessary in the case of dilute aqueous solutions of indol, for it was found that such solutions deteriorated very rapidly and if used at all had to be freshly prepared each day. If the color is found to be too intensely red to read accurately, it is desirable to add a further measured volume of chloroform until a clear pink color is obtained in 1 cc. of the extract. It is also possible to dilute the reserve test solution with water and then apply the test to the aliquot. Both methods are equally satisfactory as proved by laboratory tests. The amount of indol present in the 5 cc. test solution to be satisfactorily estimated, should not exceed about 10 micromilligrams.

TECHNIC RECOMMENDED FOR INDOL AND SKATOL DETERMINATION IN BACTERIAL CULTURES

Materials

1. Dunham's pepton solution: 10 grams pepton; 5 grams NaCl: 1000 cc. tap water. Reaction neutral (pH 7.0).

Tube 10 cc. per tube and sterilize. Since pepton occasionally contains indol, checks should always be made on the media.

2. Concentrated C. P. HCl.
3. Ethyl ether U. S. P.
4. NaOH (2.5 per cent solution) for washing ether extract in separatory funnel.
5. Dilute HCl (10 cc. concentrated C. P. HCl in 200 cc. H_2O) for washing ether extract.
6. Paradimethylaminobenzaldehyde (2 grams in 100 cc. 95 per cent alcohol).
7. $CHCl_3$—U. S. P. For extracting the indol or skatol.
8. Indol color standards (containing 0.5, 1 and 5 and 10 micromilligrams indol in 5 cc. H_2O). One micromilligram in 5 cc. is equal to a dilution of 1:5,000,000 or 0.2 part per million.
9. HCl for final indol test (600 cc. concentrated C. P. HCl plus 200 cc. distilled H_2O).
10. Distilled water.

Method

Incubate bacteria to be tested for indol production in Dunham's peptone solution, preferably at 37.5°C. for five days. Transfer contents of culture tube to 250 cc. Fry flask and wash with 40 cc. H_2O. A current of steam is passed through the culture and 100 cc. of distillate collected. If direct distillation is carried out then make up to 100 cc. and distil 75 cc. Acidify with 2 cc. concentrated HCl (2) and extract once with 50 cc. ethyl ether in a 300 cc. separatory funnel and separate ether layer. Then wash ether extract in same separatory funnel with 5 cc. of 2.5 per cent NaOH (4) followed by 5 cc. dilute HCl (5), separating the ether layer in each case. Usually these successive washings with alkali and acid are unnecessary, though they appear to remove phenols and other interfering substances. Add 10 cc. H_2O and carefully evaporate the ether on water bath. Divide the water remaining into two 5 cc. portions and test for indol as follows:

Add to one 5 cc. portion in a small colorless test tube 0.5 cc. paradimethylaminobenzaldehyde (6), then 1 cc. HCl (9). Place in boiling water for twenty seconds, shake vigorously, then place tube in ice water for one-half minute and extract with 1 cc. $CHCl_3$ (7). Compare extracted red color with that of indol standards prepared in exactly the

same manner. This test is accurate to 1:25,000,000. It is also a good though arbitrary quantitative method.

Skatol standards are not usually prepared unless the qualitative test is positive, as it is so rarely found. Skatol is indicated in this modified Ehrlich test by a pale blue color in the chloroform extract, which becomes deeper upon standing. The regular dimethylanilin test for skatol has already been described and may be applied to the reserve 5 cc. portion of the test solution.

After perfecting the technic of the indol and skatol tests as applied to bacterial cultures, several hundred determinations were made using many species of microörganisms derived from a variety of sources. Other experiments to ascertain the effect of the brand of pepton upon the speed of indol formation were also made. The several American peptones and the one imported pepton tested for indol content gave entirely negative results, though all gave positive tests for tryptophan. Witte's pepton gave by far the deepest color in the tryptophan test as well as developing more quickly than the other peptones. Slightly higher results for indol as well as more rapid formation was found using Witte's pepton, but the results were sufficiently uniform to recommend any of the domestic peptones for indol determinations. The work of Fellers, Shostrom, and Clark (1924) on hydrogen sulfide production by bacteria, showed that the several commercial peptones varied greatly as regards the formation of hydrogen sulfide. Tilley (1921) has shown this is also true in regard to indol formation. A sample of tryptophan digest submitted by a domestic firm was found to be entirely suitable for use in indol determinations. The use of such tryptic digests materially decreases the time necessary to incubate the culture. Maximum indol liberation may take place after as short a time as twenty-four hours, though for most peptones and bacteria a longer period of time appears desirable. It is known that after several days certain bacterial cultures containing indol, gradually lose their indol content. It was found that the maximum of indol liberation occurred in from forty-eight to ninety-six hours in Dunham's pepton solution, whereas a week or more was sometimes required where casein, salmon flesh or beef were

used. Table 4 contains data on the progressive accumulation of indol in cooked salmon media.

In this experiment the incubation temperature was 37°C., duplicate tubes being withdrawn at each 48 hour test period. The fact that separate tubes were used for the tests aids in explaining the variations obtained in the results. Nevertheless with the exception of culture 30-A, the results show a rather constant increase in indol content up to eight days. The blank determinations for indol upon the medium alone gave entirely negative results.

In order to determine the progressive liberation of skatol by bacteria, a skatol-liberating anaerobic clostridium isolated by one of the authors (1922), was inoculated in a 2 liter flask of Dunham's solution (pH 7) and incubated under anaerobic conditions at 35°C.

TABLE 4

Progressive accumulation of indol in bacterial cultures on cooked salmon media*

INDOL						BLANK ON MEDIA ALONE
Culture 26-A	Culture 26-B	Culture 9-A	Culture 9-B	Culture 30-A	Culture 30-B	
p.p.m.	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	
0.02	0.02	0.3	0.16	1.6	0.02	0
0.12	0.36	0.88	0.72	0.12	0.12	0
Lost	0.88	1.0	1.0	8.0	13.0	0
0.6	4.0	3.6	3.6	4.8	Lost	0

* All organisms were lactose fermenters of the colon-aerogenes group.

for thirty days, test portions of 10 cc. being withdrawn from time to time. These portions were distilled and skatol determined by the dimethylanilin test. No skatol was liberated in twenty-four hours, 20 parts per million was formed in 48 hours; 28 parts per million in seventy-two hours; and no further increase was noted even after thirty days. A repetition of the experiment gave very similar results except that an increase in skatol content in the culture from 20 to 32 parts per million occurred between the ninety-six and hundred forty-four hour testing periods.

In the course of salmon spoilage investigations, a large number of cultures of bacteria from both raw and canned salmon were examined by the modified Ehrlich test for their ability to liberate indol. Of 249 cultures obtained from raw salmon, 70 were found

to liberate indol and 3 skatol, whereas in 57 cultures (nearly all spore-forming bacteria) obtained from canned salmon only 9 liberated indol and none, skatol. Twenty-six additional cultures isolated from canned crab-meat, clams and fruit (some of which was spoiled), showed 6 cultures positive for indol and none for skatol.

A large number of known species of bacteria and other microorganisms were carefully cultured in Dunham's solution, incubated from five to seven days at 30°C., and finally tested for indol and skatol by the modified Ehrlich and other tests. One hundred eleven cultures representing 55 species were thus tested. The Ehrlich test as usually made directly upon the bacterial culture was compared with the test as performed upon the distillate. The vanillin (Steensma), Salkowski, and dimethylanilin tests were also made direct upon each culture. In the Ehrlich technic, attention was paid to the development of the pink color both in the untreated culture and in the distillate. The solubility of the pink color in chloroform was also noted in all tests made either upon the culture direct or upon the distillate. The vanillin test was performed in the usual way upon the untreated culture using hydrochloric acid. The Salkowski and dimethylanilin tests were made in the regular way also, though the former was performed only upon the untreated cultures and not upon the distillate. The results are given in table 5.

Comparing first the results obtained in table 5 by performing the Ehrlich test upon the culture direct and upon the distillate, it was found that all except four of the indol positive cultures could be detected by making the test directly upon the culture. On the other hand three slightly positive cultures obtained by the direct test were found to be negative upon testing the distillate. When large amounts of indol or skatol were present it could usually be detected by all the methods employed, though it was demonstrated to the authors' satisfaction that small amounts of indol were often overlooked when the Ehrlich test was made directly upon the culture. In about the same degree, the Salkowski and Steensma tests failed to detect small quantities of indol or skatol when the untreated culture was used for the

TABLE 5

The indol and skatol reactions of various organisms

SPECIES	NUMBER OF STRAINS TESTED	EHRLICH TEST DIRECT ON CULTURE		EHRLICH TEST MADE ON DISTILLATE		VANILLIN TEST DIRECT	SALKOWSKI TEST DIRECT
		Pink color formed	Pink color extracted by $CHCl_3$	Pink color formed	Micromilligrams indol in $CHCl_3$ extract		
Staph. pyogenes	2	−	−	+ sl.	0	−	−
Sar. lutea	2	−	−	−	0	+	−
Sar. ventriculi	1	+	+ v. sl.	−	0	++	−
Bact. enteritidis	2	+ v. sl.	−	+ v. sl.	0	−	1 −; 1 + sl.
Bact. dysenteriae Shiga	2	−	−	−	0	−	−
Bact. dysenteriae Flexner	2	+++	++	+	5	+++	1 −; 1 + sl.
Bact. paratyphosum A	4	−	−	−	0	−	−
Bact. paratyphosum B	3	++	−	++	2	+	1 +; 2 −
Bact. paratyphosum B	3	−	−	−	0	−	2 −; 1 +
Cl. skatol n. sp.	2	+++	+ blue	+	20	++	++ violet
Bact. typhosum	4	−	−	−	0	−	−
Bact. suipestifer	2	−	−	+	0	−	−
Bact. capsulatum	1	+++	−	−	0	+	+ v. sl.
Vibrio comma	1	++	++	+	13	+++	++
Bact. communior	2	+++	++	+++	20	+++	+
Bact. lactis viscosum	2	+ sl.	−	−	0	−	−
Lactob. acidi lactici	2	+ sl.	−	+ sl.	0	+	−
Bact. coli	3	+++	+	+++	15	+++	++
Bact. cloacae	2	+ sl.	−	+	0	+	+
Bact. cloacae	1	++	−	+	2	+	−
Bact. aerogenes	3	++	−	+	1.5	++	2 ++; 1 −
Bact. aerogenes	1	+	−	+ sl.	0	+ sl.	−
Bact. alkaligenes	2	−	−	−	0	−	−
Erwinia carotovora	2	+	+ sl.	+ sl.	0	+	+ v. sl.
Ps. aurescens	2	−	−	−	0	+ sl.	−

Ps. fluorescens	2	+	−	++	0	+	−
P. vulgaris	2	++	−	++	10	++	++
P. vulgaris	1	+	−	+	0	+ sl.	+ sl.
Cl. sporogenes	2	−	−	−	0	+ v. sl.	1 +; 1 −
Cl. botulinum A	2	+ sl.	−	−	0	−	−
Human feces culture	6	+++	++	++	25	+++	++
Guinea pig feces culture	6	+++	++	++	12	+++	+
Halophilic bacteria sp. ?	2	+	−	+ sl.	0	+ sl.	−
B. pseudotetanicus	1	+ sl.	−	+ sl.	0	+ sl.	−
B. fusiformis	1	−	−	−	0	−	−
B. mycoides	3	+ sl.	−	−	0	−	−
B. centrosporus	1	−	−	−	0	−	−
B. megatherium	2	+	−	−	0	+ sl.	−
Thermobacterium sp.?	1	−	−	−	0	+	−
Thermobact. thermodifferens	1	−	−	−	0	−	−
Thermobact. aerothermophilus	1	+ sl.	−	+ sl.	0	+	−
B. terminalis	1	+ sl.	−	+ v. sl.	0	+	−
Erythrob. prodigiosus	1	−	−	−	0	+ v. sl.	−
Thiobact. janiculatus	1	+	−	−	0	−	−
Sar. lactis acidi	1	−	−	−	0	+ v. sl.	−
B. cereus	2	+ sl.	−	+ sl.	0	+ v. sl.	−
B. mesentericus	2	+ sl.	−	−	0	+ sl.	−
B. circulans	1	+	−	+	0	+	−
B. cohaerens	1	−	−	+	0	+ sl.	−
B. albolactis	1	+ sl.	−	−	0	+ sl.	−
B. subtilis	2	+	+ sl.	++	0	++	1 −; 1 + sl.
B. flexus	1	−	−	−	0	+	−
B. vulgatus	2	+	−	−	0	+ sl.	−
Sacch. ellipsoideus	1	+ sl.	−	−	0	−	−
Saccharomyces sp.?	1	−	−	−	0	+ sl.	−
Aspergillus flavus	1	−	−	−	0	−	−
Penicillium sp.?	1	+ sl.	−	−	0	+ v. sl.	−
Actinomyces griseus	1	−	−	−	0	−	−

+, positive; −, negative.

test. The turbidity, varying hydrogen-ion concentrations and interfering substances probably account for most of the inaccuracies obtained by testing the untreated culture. None of the 18 aerobic spore-forming bacteria tested, liberated indol or skatol; similarly, 4 cocci, 2 yeasts and 2 molds liberated no indol from pepton under the conditions of the experiment. The principal indol liberating bacteria are those of the colon-aerogenes and proteus groups. Several organisms like *P. vulgaris*, *Bact. dysenteriae*, *Bact. aerogenes*, and *Bact. paratyphosum* B apparently vary as to their ability to form indol from pepton, some cultures and sub-species being positive and others negative. All cultures were tested for skatol by the dimethylanilin test. All were negative with the exception of *Cl. skatol* n. sp. which was strongly positive.

On the whole the vanillin (Steensma) test was least satisfactory for it gave many positive results which could not be confirmed by other tests. One reason for this is that the color produced is read directly without extraction by any solvent as in the Ehrlich test. It is also believed that interfering substances are often present in bacterial cultures which give orange or red colors with the reagent, thus making the test less accurate and reliable. The chief use of the Steensma vanillin test is in its confirmatory value. When used alone it is unreliable. The Salkowski test is suitable where large amounts of indol are present, but it is not sensitive enough for ordinary purposes. Furthermore the production of a color closely resembling the indol-red color results when acid alone or acid plus sodium nitrite are added to indol-acetic and indol-propionic acids. When the Salkowski test is performed upon the distillate, however, it is much more sensitive and reliable, though it hardly compares with the modified Ehrlich technic in these respects. It is recommended mainly as a confirmatory test.

SUMMARY

1. A critical review of the literature on the indol reaction as applied to bacterial cultures has been compiled.

2. The Ehrlich, vanillin, Salkowski, β-naphtho-quinone, dimethylanilin, Konto, nitroprusside, Gnezda, furfural, glyoxylic

acid, methyl alcohol, Baudisch and other tests for indol and skatol were carefully studied and compared for reliability, accuracy, simplicity and practicability.

3. The Ehrlich test was found to give the best results. A suitable technic for performing the test was worked out with excellent results when applied to bacterial cultures. It was found that not only the Ehrlich but most other indol and skatol tests when applied directly to the liquid medium did not yield consistent nor accurate results particularly when only small amounts of indol or skatol were present.

4. It was found necessary to distil the culture and make the tests upon the distillate. By the use of standards prepared from pure indol or skatol the technic may be made quantitative. Small quantities of indol may be detected by this method which are entirely overlooked when testing the culture direct. Indol acetic acid and other interfering substances which are often present in bacterial cultures do not seriously affect this test. Steam or direct distillation may be used. Satisfactory recoveries of indol are obtained by either method of distillation. Precautions in carrying out the technic were carefully worked out and must be closely followed if quantitative results are sought.

5. The test is accurate to 1 part to 25,000,000.

6. In the characterization of bacterial species it is recommended that the indol and skatol tests be applied quantitatively and following an approved technic. The present chaos surrounding the indol test in bacteriology is largely attributable to the diversity of methods, tests, mediums and many other variable factors.

7. The dimethylanilin test for skatol as described in this paper is recommended as the most satisfactory reaction for this substance. The test should be made upon the distillate otherwise it may give negative results. Of 53 species tested for skatol only one, *Cl. skatol* n. sp. gave positive results. The Ehrlich reaction also serves as a valuable indicator of skatol.

8. It is not safe to rely upon the production of a red color in the culture tube as a positive indol test regardless as to whether the test be the Ehrlich, vanillin or Salkowski, unless the color is

pronounced and soluble in chloroform. Although distillation by steam or directly requires a little time, yet it has been found that once the apparatus is set up, the tests may be run very expeditiously. It is believed that the quantitative and reliable nature of the results obtained are more than sufficient justification for the use of such a method.

REFERENCES

BAEYER 1870 Ann. Chem. u. Pharm. Suppl., **7**, 56.
BARTHEL 1921 Jour. Bacteriol., **6**, 85.
BAUDET 1913 Folia microbiol., Delft., **2**, 261.
BAUDISCH 1915 Zeitschr. physiol. Chem., **94**, 132.
BERGHEIM 1917 Jour. Biol. Chem., **32**, 17.
BLUMENTHAL 1909 Biochem. Zeitschr., **19**, 521.
BOEHME 1906 Centrbl. Bakt., Orig., **40**, 129.
CANNON 1916 Jour. Bacteriol., **1**, 535.
CHEN AND RETTGER 1920 Jour. Bacteriol., **5**, 253.
CLOUGH 1922 Publ. Puget Sound Biol. Sta., **3**, 219.
CRISAFULLI 1895 Revista d'Igiene e Sanitá publica, **6**, 198.
CROSSONINI 1910 Arch. Hyg., **72**, 161.
DAKIN 1906 Jour. Biol. Chem., **2**, 289 .
DENIGES 1908 Repert. pharm. (3), **20**, 160 and 161.
EHRLICH 1901 Berl. med. Wochenschr., **1**, 151.
ESCALLON AND SICRE 1906 Compt. rend. Soc. Biol., **65**, 507.
FELLERS 1922 Bact. Abs., **7**, 351.
FELLERS, SHOSTROM AND CLARK 1924 Jour. Bacteriol., **9**, 235.
FREUND 1922 Centralbl. Bakt., Orig., **88**, 9.
FRIEBER 1921 Centralbl. Bakt. Orig., **64**, 65.
GNEZDA 1899 Compt. rend. Acad. Sci., **128**, 1584.
GORÉ 1921 Indian Jour. Med. Res., **8**, 505.
GORTER AND DEGRAEFF 1908 Pharm. Weekbl., **45**, 842.
HAENEN 1905 Arch. Intern. de Pharmacodynamie et de Therapie, **15**, 255.
HAWK 1918 Practical Physiological Chemistry, 6th ed., 222.
HERTER 1905 Jour. Biol. Chem., **1**, 251 and 255.
HERTER 1908 Jour. Biol. Chem., **4**, 253.
HERTER AND FOSTER 1905 Jour. Biol. Chem., **2**, 267 and 289.
HOLMAN AND GONZALES 1923 Jour. Bacteriol., **8**, 577.
HOMER 1914 Biochem. Jour., **7**, 101.
HOMER 1915 Jour. Biol. Chem., **22**, 345.
HOMER 1916 Jour. Hyg., **15**, 401.
HOPKINS AND COLE 1903 Jour. Physiol., **29**, 451.
HOROWITZ 1916 Annal. Inst. Pasteur, **30**, 307.
JORDAN 1919 Jour. Amer. Med. Assoc., **72**, 1542.
KITASATO 1899 Zeitschr. Hyg. u. Infektionsk., **7**, 515.
KLIGLER 1913 Proc. Soc. Exper. Biol. Med., **11**, 12.

KLIGLER 1914 Jour. Inf. Dis., **14**, 81.
KOLLE AND WASSERMANN 1913 Handbuch der pathogenen Mikroorganismen, 2d Ed. Jena.
KONTO 1906 Zeitschr. physiol. Chem., **48**, 185.
LEVINE AND LINTON 1924 Amer. Jour. Public Health, **14**, 99.
LEWIS 1915 Jour. Path. and Bact., **19**, 429.
MALONE AND GORÉ 1921 Indian Jour. Med. Res., **8**, 490.
MORELLI 1908 Rev. Crit. de Clin. Med. Firenze, no. 5.
NELSON 1916 Jour. Biol. Chem., **24**, 527 and 533.
NENCKI 1875 Berl. Chem. Ges., **8**, 1517.
NENCKI 1901 Berl. Chem. Ges., **34**, 201.
NONOTTE AND DEMANCHE 1908 Compt. rend. Soc. de Biol., **44**, 658.
NORTON AND SAWYER 1921 Jour. Bacteriol., **6**, 471.
PITTALUGA 1908 Bol. Inst. nac. de hyg. de Alfonso XIII. Madrid. No. 13.
RIVAS 1912 Centralbl. Bakt. Orig., **63**, 547.
RÖHMANN 1908 Biochemie, 449. Julius Springer, Berlin.
SALKOWSKI 1883 Zeitschr. physiol. Chem., **8**, 425.
SALKOWSKI 1919 Biochem. Zeitschr., **97**, 123.
SASAKI 1909 Biochem. Zeitschr., **23**, 402.
SCHMIDT 1903 Munch. med. Wochenschr., **50**, no. 17.
STEENSMA 1906 Zeitschr. physiol. Chem., **47**, 25.
STEENSMA 1906 Centralbl. Bakt. Orig., **41**, 295.
TELLE AND HÜBER 1911 Centralbl. Bakt. Orig., **56**, 70.
TILLEY 1921 Amer. Jour. Public Health, **11**, 834.
TOBEY 1906 Jour. Med. Research, **15**, 301 and 305.
VAN LOGHEM AND VAN LOGHEM-POUW 1912 Centralbl. Bakt. Abst. 1, **66**, 19.
WEEHUIZEN 1908 Pharm. Weekblad., **45**, 1325. Also Chem. Abstracts, **3**, 660.
WENNER AND RETTGER 1919 Jour. Bacteriol., **4**, 331.
ZIPFEL 1912 Centralbl. Bakt. Orig., **64**, 65.
ZOLLER 1920 Jour. Biol. Chem., **41**, 25 and 37.

CONCERNING THE PHYSIOLOGY OF THIOBACILLUS THIOOXIDANS, AN AUTOTROPHIC BACTERIUM OXIDIZING SULFUR UNDER ACID CONDITIONS[1]

ROBERT L. STARKEY

From the Department of Soil Chemistry and Bacteriology, New Jersey Agricultural Experiment Stations, New Brunswick, New Jersey

Received for publication July 21, 1924

INTRODUCTION

Since 1921 numerous researches from this laboratory (Lipman, Waksman, and Joffe, 1921; Waksman and Joffe, 1922 a and b; Joffe, 1922 a, b and c; Waksman, 1922 b, c and d; Waksman and others, 1923; Waksman and Starkey, 1922, 1923; Allison, 1923; Lipman, 1923) have been concerned with the physiology of an unusual autotrophic sulfur-oxidizing bacterium called *Thiobacillus thiooxidans*,[2] first isolated by Waksman and Joffe (1921 a and b). This is but one of numerous sulfur-oxidizing bacteria found in nature some of which are colorless while others are colored, filamentous, or short rod forms. Some secrete elementary sulfur within the cells and others do not (Düggeli, 1919; Waksman, 1922 a).

Thiobacillus thiooxidans Waksman and Joffe, is one of several non-filamentous bacteria not accumulating elementary sulfur within the cells. *Thiobacillus thioparus* Beijerinck, oxidizes thiosulfate, tetrathionate, sulfides, and sulfur (Nathansohn, 1902; Beijerinck, 1904 a and b; Jacobsen, 1912, 1914). Sulfur is quantitatively oxidized to sulfate from sulfur, but some sulfur is

[1] Paper No. 189 of the Journal Series of the New Jersey Agricultural Experiment Stations, Department of Soil Chemistry and Bacteriology.

[2] In a previous paper (Waksman and Starkey, 1923) the generic name *Sulfomonas* was used. Since that paper was written attention has been called to the fact that Orla-Jensen's suggestion for changing the name of *Thiobacillus* to *Sulfomonas* was not justified, therefore the term *Thiobacillus* should be retained.

precipitated during the oxidation of thiosulfate, tetrathionate, or sulfides. *Thiobacillus denitrificans* Beijerinck, oxidizes thiosulfate and sulfur under anaerobic conditions using nitrate as the source of oxygen (Beijerinck, 1904 a and b; Lieske, 1912; Gehring, 1915). Both of these organisms are strictly autotrophic; that is, development is normal where no organic compounds are introduced into the media and the sole source of carbon for metabolic processes is carbon dioxide (dissolved). A species of *Thiobacillus* studied by Trautwein (1921, 1924) and Klein and Limberger (1923) closely resembles *Thiobacillus denitrificans* in its autotrophic nutrition, but lives as well heterotrophically. All of the above forms other than *Thiobacillus thiooxidans* develop under neutral or alkaline conditions. Unlike any of the other forms, *Thiobacillus thiooxidans* makes no development in synthetic media under neutral or alkaline conditions, but shows normal rapid growth at an extremely acid reaction. When freshly isolated, however, it may grow in neutral and even slightly alkaline media. In fact, *this bacterium is distinctive in that it is able not only to tolerate, but to produce higher concentrations of acid than any other living organism yet known.* Further, this acid is mineral and not organic.

With the exception of Brown's studies (1923) no work has been reported outside of these laboratories concerning oxidation of sulfur by pure cultures of organisms similar to *Thiobacillus thiooxidans.* If not identical with this bacterium, the organism studied by Brown is very closely related. Physiologically, they react qualitatively in very much the same manner. The main difference is apparently the appearance of growth in solution media. *Thiobacillus thiooxidans* shows a characteristic greyish turbidity even in three days which increases in intensity as growth proceeds. The organism studied by Brown showed practically no turbidity. It may be demonstrated later that this organism is another strain of the one isolated at this laboratory. At least, Brown's organism much more closely resembled *Thiobacillus thiooxidans* than any of the other sulfur organisms reported to date.

HISTORICAL

Thiobacillus thiooxidans develops only on strongly elective media using elementary sulfur or sodium thiosulfate as sources of energy. The presence of some organic compounds in the medium is not injurious even in considerable amounts and may even be stimulating, but growth does not occur in organic media in the absence of inorganic sulfur (Waksman and Starkey, 1923). As oxidation of the sulfur proceeds, sulfuric acid accumulates and the medium increases in acidity. When calcium phosphate is added in the medium, crystals of calcium sulfate (gypsum) are produced in abundance, as growth progresses, by the reaction between the phosphate and the sulfuric acid produced. It has been definitely demonstrated (Waksman and Starkey, 1923) that the oxidation of sulfur to sulfate is quantitative and no intermediate oxidation products of sulfur accumulate.

The source of energy for growth is principally the oxidation of elementary sulfur, but thiosulfate is also oxidized. Oxidation of sulfur is exothermal and considerable energy is liberated by the formation of sulfuric acid.

$$\underset{2 \times 69.0}{2S + 2H_2O + 3O_2} \longrightarrow \underset{2 \times 210.8}{2H_2SO_4} + 283.6 \text{ Cal.} \qquad \text{(I)}$$

From this reaction, 141.8 Cal. are liberated from one gram atom of sulfur.

In the presence of small amounts of sodium thiosulfate (0.5 per cent) oxidation proceeds by the following reaction (Waksman and Starkey, 1923):

$$\underset{258.0}{Na_2S_2O_3} + \underset{69.0}{2O_2} + H_2O \longrightarrow \underset{332.6}{Na_2SO_4} + \underset{210.8}{H_2SO_4} + 216.4 \text{ Cal.} \qquad \text{(II)}$$

According to this reaction, 108.2 calories are liberated from one gram atom of sulfur in the form of thiosulphate.

Oxidation is aerobic and the source of carbon is carbon dioxide from the atmosphere. Consequently, considerable amounts of oxygen and carbon dioxide are indispensable to growth and oxidation.

The organism is remarkably tolerant to high concentrations of certain inorganic salts (Waksman and Starkey, 1923). Magnesium sulfate did not inhibit oxidation even in concentration of 20 per cent. Sodium and calcium were somewhat more toxic, but some oxidation took place in concentrations of 10 per cent of salts of both of these cations. As good growth occurred at 1 per cent as in the absence of these salts and oxidation was rapid at 5 per cent. Sulfates and chlorides are injurious only at high concentrations.

EXPERIMENTAL METHODS

Cultural. In the following investigations a solution medium of the following composition has been used unless modifications are indicated in the text:

Distilled water	100 cc.
Sulfur or $Na_2S_2O_3 \cdot 5H_2O$	1.0 gram. 0.5 gram
$(NH_4)_2 SO_4$	0.02 to 0.04 gram
$KH_2 PO_4$	0.3 to 0.4 gram
$Ca Cl_2$	0.025 gram
$Mg SO_4$	0.05 gram
$Fe SO_4$	0.001 gram

The sulfur was weighed separately for each flask and the mineral solution added to it. The flasks of media were sterilized in flowing steam for one-half hour on three consecutive days. When used as the source of energy in place of the sulfur, the thiosulfate was sterilized separately in solution and added to the individual flasks of sterile mineral solution in the desired amounts. For the experiments on carbon assimilation particular precautions were taken to free the media from contaminating carbon. The water used for preparing such media was distilled with chromic acid and barium hydroxide to remove the carbon. The glassware was cleaned with a solution of potassium bichromate in sulfuric acid. In most cases, 250 cc. Erlenmeyer flasks containing 100 cc. of the medium were used. For the carbon assimilation studies, 1 liter Erlenmeyer flasks containing 300 cc. of medium were used. All cultures were incubated at 26 to 28°C.

Analytical. The *reaction* changes in the media were followed by measuring the acidity by titration and determining the hydrogen-ion concentration. For the first method, 5 cc. of the culture were titrated with 0.1 N sodium hydroxide, using phenolphthalein as the indicator. "Titer" designates the cubic centimeters of 0.1 N sodium hydroxide to neutralize 5 cc. of culture. The hydrogen-ion concentration was determined by means of Clark and Lubs' indicators. Comparisons of the colors of the cultural solutions were made with the colors of a series of standard buffer solutions. The hydrogen-ion concentration has not proved an accurate measurement of oxidation in the cultures. This is due to the fact that the reaction rapidly drops below pH 1.2 and at the acid range slight changes in the pH indicate considerable changes in acidity. In most instances titration and sulfate determinations have been made in preference to determinations of the hydrogen ions.

Sulfates were determined by the usual gravimetric method. The sulfates were precipitated in a hot solution with barium chloride. This precipitate was filtered off, washed, ignited, and weighed.

Total carbon in the media was determined by a wet combustion method explained in detail in another place (Waksman and Starkey, 1923). The carbon in the medium was oxidized to carbon dioxide by permanganate in an acid solution, aerated into standard barium hydroxide, and determined by titration with oxalic acid.

EXPERIMENTAL RESULTS

Rate of oxidation of sulfur at different stages in the process

The course of the reaction of the cultures in ten one-liter flasks was followed by determining the acidity by titration. At intervals, 25 cc. of the culture were withdrawn from each flask. The portions from the ten flasks were mixed together, put through filter paper and divided into 60 cc. portions in 100 cc. Erlenmeyer flasks each containing 1 gram of sulfur. The course of the reaction was then followed in the cultures in these small flasks at intervals of a day or more. The culture in the 1 liter flasks is

TABLE 1

Rate of oxidation of sulfur in solution cultures

DAYS OF INCUBATION	TITER OF MOTHER CULTURE	TITER OF SUB-CULTURES							
		I	II	III	IV	V	VI	VII	VIII
0	1.05								
1	1.09								
2	1.28	1.25							
3	2.04	1.48							
4	3.28	2.82	3.40						
5	4.39	5.02	4.17						
6	5.54	7.28	5.90	5.65					
7	6.69	9.67	7.78	6.47					
8	7.72		9.80	7.78					
9	8.50	14.25	12.05	9.33	8.40				
10				11.67	9.08				
11	9.93	16.90	16.20	13.07	9.97				
12					12.27				
13	10.80	19.67	18.82	16.48	13.35	10.75			
14					15.37	10.88			
15									
16	11.43		21.97	18.90	17.55	12.97			
17					19.00	14.92			
18	11.91			21.03	19.87	16.60	11.80		
19						18.25	12.07		
20						18.97	12.82		
21						20.12	14.55		
22					24.97		15.22		
23							16.57		
24	12.86					22.08		13.10	
25									
26							17.83	13.38	
27									
28							19.20	16.37	
29								17.72	
30							20.77		
31								19.48	
32								20.32	
33									
34								21.93	
35									
36	14.34							23.47	14.40
37									14.55
38									14.90
39									16.38
40									17.68
41									18.93
42									
43									20.38
44									
45									21.80
46									
47									
48									24.23

termed "mother culture" and the cultures in the small flasks "sub-cultures." Eight series of sub-cultures were started from the mother cultures from two to thirty-six days after the mother cultures were first inoculated.

If oxidation proceeded with equal rapidity in all of the sub-cultures per unit time it could be concluded that the oxidation of sulfur proceeds with equal rapidity throughout the process. Further, if the velocity of oxidation was not the same in all the sub-cultures the stages of maximum and minimum velocity would

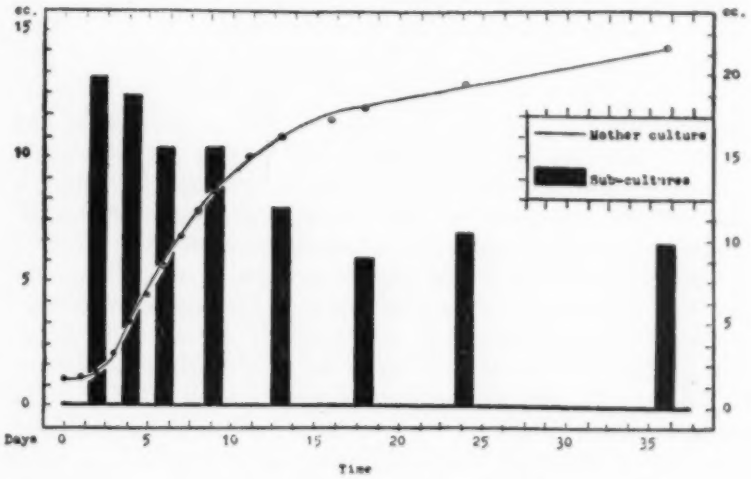


FIG. 1. VELOCITY OF OXIDATION OF SULFUR AT DIFFERENT STAGES

be indicated. The data are presented in table 1 and figure 1. Only averages of the mother and sub-cultures for each interval are given. In figure 1, the curve represents the course of oxidation in the mother cultures. The block figures represent the increases in titers of the sub-cultures for a period of incubation of twelve days.

It is apparent that the course of oxidation in the mother cultures takes the form of a growth curve when expressed graphically. This was brought out earlier by Waksman and Joffe (1922 a) The block figures are inserted on the curve at the intervals in the

process when the sub-cultures were started from the mother culture. The amount of oxidation per unit of time is greatest in the early stages of the process following a short lag period and decreases as the culture ages, the most rapid oxidation occurring one or two days after the culture becomes inoculated. This is not apparently due to any attenuation of the organism or to the accumulation of any toxic organic metabolic products, but rather to the accumulation of sulfuric acid. This conclusion is further substantiated by other results (Waksman and Starkey, 1923) which indicate that oxidation is retarded in media initially containing considerable quantities of sulfuric acid.

Sub-cultures from the mother culture, after oxidation had almost ceased in the latter, gave considerable oxidation due to the introduction of fresh sulfur. As oxidation progresses in any culture the sulfur becomes submerged in the medium, practically all of the sulfur sinking after two weeks or more. Oxidation is considerably retarded when the sulfur is not suspended on the surface of the medium. Consequently, the addition of fresh sulfur to the surface accelerates the process of oxidation. The factor of variability in the amounts of sulfur suspended on the surface at different stages of the process is eliminated by introducing the same amount of sulfur in each of the sub-cultures. It can be concluded that oxidation is much more rapid the first week than after that time. All of the sub-cultures reached about the same acidity after incubation for twelve days having titers from 19.67 to 24.97. This would be equivalent to 0.4 to 0.5 normal acid. If the source of energy (sulfur) could be continually supplied in an available state and the end product (sulfuric acid) removed, it is probable that oxidation would proceed uniformly for a considerable period of time.

Influence of initial acid concentration on oxidation and growth

Oxidation proceeds most rapidly between pH 2.5 and 3.5 (Waksman and Starkey, 1923) although oxidation of sulfur is very rapid over a very much wider range. Above 6.0 there is practically no oxidation of sulfur in the culture medium by this bacterium. Oxidation was feeble in a medium initially con-

taining 5 per cent sulfuric acid and in the presence of 10 per cent acid oxidation did not start. However, in cultures initially receiving no addition of sulfuric acid, as high as 10.2 per cent or 2.08 normal acid has been produced by the organism. As the organism increases the acidity of the medium it may become adjusted to the high concentrations. When the organism is inoculated from a relatively dilute culture to the concentrated medium it may not survive the shock.

With regard to the tolerance of the organism to extremely high acid concentration certain statements by Falk (1923) may be applicable.

> There are probably very few groups of animal or plant forms . . . . whose physiologic indifference to marked changes in osmotic pressure equals that of bacteria. It is perhaps safe to say that within limits which would be fabulous and astonishing for nearly any other form of actively metabolising protoplasm, the bacteria are commonly indifferent to changes in osmotic pressure. . . . . The indications seem clear that we do not know the osmotic pressure of bacterial protoplasm, whether it has a fixed value for any one or for different species, or whether it has true physiological significance.

It has been demonstrated (Waksman and Starkey, 1923) that there is a constant relationship between the amount of sulfur oxidized to sulfate and the amount of carbon assimilated from carbon dioxide to organic forms by the organism by the energy so liberated. This proportion has been expressed as the sulfur-carbon ratio and was found to be 31.82 under conditions favorable for growth. It has further been estimated that under these conditions about 6.7 per cent of the energy liberated from the sulfur had been used for synthesis of the organic compounds from carbon dioxide.

In table 2 results are presented concerning the assimilation of carbon by the organism in media containing relatively high initial concentrations of sulfuric acid. There was considerable oxidation of sulfur in media initially containing both 1 and 5 per cent sulfuric acid. There was somewhat less oxidation and considerably less carbon assimilation at the higher concentration.

TABLE 2

Carbon assimilation in the presence of high initial concentration of sulfuric acid

DAYS OF INCUBATION	TREATMENT	REACTION	TITER	TOTAL C, AS C IN 100 CC.				TOTAL SULFATE, AS S IN 100 CC.				S/C	
				1	2	Average	After subtracting control	1	2	Average	After subtracting control	Per culture	Average
		pH		*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*		
37	1 per cent control	−1.2	12.00	2.802	3.290	3.046		376.27	382.45	379.36			
18	1 per cent inoculated	−1.2	28.00	16.033	15.451	15.742	12.696	816.40	842.48	829.44	450.08	35.45	
25		−1.2	27.20	16.493	15.669	16.081	13.035	857.86	869.82	863.84	484.48	37.17	
33		−1.2	26.70	14.780	16.314	15.547	12.501	850.04	839.46	844.75	465.39	37.23	36.62
36	5 per cent control	−1.2	55.10	2.534	—	2.534		1,795.6	1,766.6	1,781.1			
20	5 per cent inoculated	−1.2	68.00	9.454	9.225	9.340	6.806	2,020.2	2,058.2	2,039.2	258.1	37.92	
26		−1.2	66.70	11.021	—	11.021	8.478	2,179.0	2,138.9	2,159.0	377.9	44.58	
34		−1.2	67.85	11.820	—	11.820	9.286	2,217.1	2,207.3	2,212.2	431.1	46.42	42.97

At the lower concentration the average sulfur-carbon ratio was 36.62. In 5 per cent acid the ratio was somewhat higher, 42.97. Both of these ratios are considerably higher than for oxidation in the normal medium. A high ratio indicates less economical utilization of the energy liberated. It would be expected that the ratio would therefore be high under unfavorable conditions. It has been shown that conditions are unfavorable in the presence of considerable amounts of acid, more so at the higher concentrations. The high sulfur-carbon ratios under these conditions seem to bear out this point.

Oxidation and growth in the presence of different amounts of thiosulfate

Large amounts of sulfur in the medium have no injurious effects on oxidation but greatly accelerate the process instead (Waksman and Starkey, 1923). The relationships observed indicate that the velocity of oxidation is closely proportional to the logarithm of the amount of sulfur present. Since sulfur is only very slightly soluble in water, small amounts of sulfur would create a saturated solution. The increase in velocity of oxidation with increasing amounts of sulfur in the medium is probably due to the increased surface of the sulfur exposed to the organisms. The portion of the total surface of the sulfur particles exposed to the solution is greatest when the amount of sulfur added is small and is not proportional to the amount of sulfur in the medium. This may explain why the increase in velocity of oxidation is not proportional to the amount of sulfur added.

In the presence of soluble sources of energy the relationships are quite different. This is evidenced in cultures containing sodium thiosulfate as the source of energy (table 3). Oxidation is rapid up to a certain concentration beyond which oxidation ceases. Oxidation proceeds quantitatively to sulfate in the presence of moderate concentrations of thiosulfate (0.5 per cent) and this is accompanied by a corresponding increase in acidity (Waksman and Starkey, 1923). In media containing 1 per cent or more of sodium thiosulfate, intermediate oxidation products

accumulate. Oxidation by *Thiobacillus thiooxidans* apparently proceeds directly to sulfate and tends to increase the acidity (reaction II, page 137). Sulfur is chemically precipitated from thiosulfate by mineral acids. Such a precipitation has been repeatedly observed in cultures containing high concentrations of thiosulfate. In the precipitation of this sulfur, sodium ions are released from the thiosulfate, the free acid is neutralized, and the

TABLE 3

Concentration of thiosulfate and oxidation of sulfur

CONCENTRATION OF THIOSULFATE	TITER AFTER			pH AFTER		S AS SULFATE AFTER			
	7 days	21 days	36 days	7 days	36 days	7 days	After subtracting control	36 days	After subtracting control
per cent						*mgm.*	*mgm.*	*mgm.*	*mgm.*
0.10*	1.20			4.6		18.123			
0.10	1.60	1.75	1.90	2.5		45.309	27.186		
0.10	1.60	1.75	1.90	2.5		45.584	27.461		
0.25*	1.20			4.7		21.007			
0.25	1.30	2.50	2.75	4.2		41.190	20.183		
0.25	1.20	2.50	2.70	4.5		37.071	16.064		
1.00*	1.20			4.8		27.460			
1.00	1.20	6.20	6.90	4.6	−1.2	82.380	61.373	328.146	300.686
1.00	1.20	5.70	6.85	4.6	−1.2	83.203	62.196	336.385	308.925
3.00*	1.15			4.8		41.190			
3.00	1.20	1.25	13.10	4.6	−1.2	285.313	257.853	820.367	779.177
3.00	1.20	1.25	12.30	4.6	−1.2	112.585	85.125	806.640	765.450
10.00*	1.10			5.2					
10.00	1.15	1.20	1.30	5.2					
10.00	1.15	1.20	1.25	5.2					
25.00*	0.90			5.4					
25.00	0.95	0.90	1.05	5.4					
25.00	0.90	0.90	0.95	5.4					

* Uninoculated controls; all others inoculated.

reaction of the medium does not become acid. As shown in table 3 considerable sulfate is produced at rather high concentrations of thiosulfate and there is no change in reaction, but precipitation of sulfur occurs. Later, after the thiosulfate has all been decomposed, the reaction becomes acid by oxidation of the sulfur precipitated in the medium. It was only after twenty-one days that any appreciable change in reaction took place in the cultures

containing 3 per cent thiosulfate although considerable sulfate had been produced. The rate of the disappearance of thiosulfate in the cultures is, therefore, much more rapid than is the increase in acidity or production of sulfate. Eventually, all of the sulfur originally contained in the thiosulfate appears as sulfate. No growth took place in media containing 10 per cent thiosulfate although good oxidation occurred at the concentration of 3 per cent.

Sulfur precipitation has been repeatedly noticed during the process of oxidation of thiosulfate by *Thiobacillus thioparus*, Nathansohn (1902) believed that this precipitation was the result of secondary reactions, but Beijerinck (1904 a and b) emphasized that it was a result of primary oxidation. He, therefore, concluded that the liberation of energy from thiosulfate must occur outside of the cells of the bacteria. The fact that the sulfur is precipitated without the cell in itself would seem to indicate that the precipitation was the result of some secondary reaction. It seems unlikely that an organism could utilize the energy liberated under such conditions. Whether this secondary reaction is the same in cultures of both *Thiobacillus thioparus* and *Thiobacillus thiooxidans* is not certain since the media have different reactions, media for *Thiobacillus thioparus* being alkaline.

Lieske (1912) has determined that oxidation of sulfur and sulfur compounds by denitrifying organisms occurs in stages and is not direct. This evidence itself indicates that the reactions involved in the oxidation processes are not so simple as might be supposed, judging from the end products of the rapid transformations.

The results concerned with the assimilation of carbon in the presence of 0.5 and 1 per cent thiosulfate are presented in table 4. In the presence of 0.5 per cent thiosulfate the reaction became acid but in cultures containing 1 per cent of the salt the reaction did not change appreciably in nineteen days. There was an accumulation of sulfate in both instances. The sulfur-carbon ratios varied to such an extent that the differences between the averages of the ratios at the two concentrations was not significant. The average ratios in the presence of 0.5 and 1 per cent thiosulfate were 64.60 and 51.53 respectively. These are nearly twice as great as in the oxidation of sulfur.

TABLE 4

Course of oxidation of thiosulfate and assimilation of carbon

DAYS OF INCUBATION	TREATMENT	REACTION	TITER	TOTAL C, AS C IN 100 CC.				TOTAL SULFATE, AS S IN 100 CC.				S/C	
				1	2	Average	After subtracting control	1	2	Average	After subtracting control	Per culture	Average
		pH		*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*		
6	0.5 per cent	5.0	0.70	1.662	—			11.372	12.855				
24	control	4.8	0.70	1.998	1.705	1.788		16.700	14.558	13.871			
16	0.5 per cent	1.9	2.65	3.577	3.284	3.431	1.643	131.024	130.530	130.777	116.906	70.55	
20	inoculated	1.8	2.68	3.965	3.764	3.865	2.077	135.419	135.913	135.666	121.795	58.64	64.60
13	1 per cent	4.8	1.10	—	—			20.945	20.258				
14	control	4.8	1.10	1.635	1.665	1.650		18.884	19.228	19.829			
7	1 per cent	4.6	1.15	2.014	2.014	2.014	0.364	40.173	39.492	39.833	20.004	54.96	
13	inoculated	4.6	1.20	3.132	3.009	3.071	1.421	76.223	77.254	76.739	56.910	40.05	
19		4.8	1.20	3.422	3.223	3.323	1.673	118.114	120.858	119.486	99.657	59.57	51.53

According to reaction II on page 137, 108.2 calories are liberated for each gram atom of sulfur in the form of thiosulfate which is oxidized to sulfate. Assuming that oxidation proceeds according to this reaction, the proportion of the total energy available, which is utilized to assimilate carbon, was 4.31 per cent in the lower concentration and 5.40 per cent in the presence of 1 per cent thiosulfate. According to these figures, the energy utilization from thiosulfate is much less economical than from sulfur.

Lieske (1912) found that 10.9 mgm. of carbon were assimilated by *Thiobacillus denitrificans* with the oxidation of 1 gram of $Na_2S_2O_3 \cdot 5H_2O$ to sulfate. The sulfur-carbon ratio in this instance would be 23.7 (?). The economy of the assimilation of the energy liberated by the oxidation of sodium thiosulfate according to this ratio would be considerably greater than the economy of *Thiobacillus thiooxidans*. Further, *Thiobacillus thiooxidans* utilizes atmospheric oxygen while *Thiobacillus denitrificans* uses oxygen from nitrates. Considerable energy would be consumed in the reduction of nitrate. From the results of Lieske, the denitrifying sulfur organism is comparatively economical in its utilization of the energy available for carbon assimilation.

Influence of concentration of phosphates on oxidation and growth

The organism seems quite tolerant to rather high concentrations of phosphate (Waksman and Starkey, 1923). Oxidation was not depressed in the presence of 3 per cent sodium or potassium phosphate. The concentration limiting oxidation was not found at 7 per cent although oxidation was considerably depressed. Even 10 per cent calcium phosphate did not completely inhibit oxidation.

The economy of the assimilation of carbon in media containing large amounts of soluble phosphates was investigated (table 5). The medium containing 0.3 per cent phosphate is the normal medium. At all concentrations (0.3, 1.36, and 5.45 per cent) oxidation and growth were rapid as indicated by the acidity, sulfate production, and carbon assimilation. In the normal medium the sulfur-carbon ratio was practically the same as determined earlier, 31.75. In the 1.36 per cent solution the ratio

TABLE 5

Sulfur oxidation and carbon assimilation in the presence of concentrations of KH_2PO_4

DAYS OF INCUBATION	TREATMENT	REACTION	TITER	TOTAL C, AS C IN 100 CC.				TOTAL SULFATE, AS S IN 100 CC				S/C	
				1	2	Average	After subtracting control	1	2	Average	After subtracting control	Per culture	Average
		pH		*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*		
0	0.3 per cent control	4.6	1.15	3.694	2.948	3.321		22.32	—	22.32			
13	0.3 per cent inoculated	−1.2	17.55	19.202	—	19.202	15.881	506.19	501.72	503.96	481.64	30.33	
14		−1.2	15.90	15.416	17.526	16.471	13.150	455.23	460.38	457.81	435.49	33.12	31.73
1	1.36 per cent control	4.4	5.20	2.671	3.215	2.943		20.26	—	20.26			
17	1.36 per cent inoculated	−1.2	19.95	15.925	15.099	15.512	12.569	442.87	440.13	441.50	421.24	33.51	
21		1.4	21.20	17.470	17.730	17.600	14.657	535.23	530.98	533.11	512.85	34.99	
29		1.2	21.30	17.838	18.431	18.135	15.192	527.12	532.49	529.82	509.56	33.54	34.01
2	5.45 per cent control	4.4	20.60	2.723	2.688	2.705		26.09	—	26.09			
10	5.45 per cent inoculated	3.3	25.50	5.620	5.923	5.772	3.067	128.47	127.85	128.16	102.07	33.29	
19		2.4	29.10	9.041	9.668	9.355	6.650	223.50	227.27	225.39	199.30	29.97	
28		1.8	32.10	15.368	14.335	14.852	12.147	392.27	—	392.27	366.18	30.15	31.14

was somewhat higher, 34.01. It was expected that the ratio would be still greater in 5.45 per cent phosphate since oxidation is depressed at that concentration as shown in table 5 by the comparative amounts of acid and sulfate produced. The ratio was 31.14, practically the same as in the lower concentrations of phosphate. Apparently phosphates do not appreciably alter the normal metabolism of the organism even in 5.5 per cent concentration.

Since phosphates did not seem injurious, Joffe (1922 a, 1923 a) was of the opinion that gypsum was the factor which retarded oxidation in composts. This seems quite improbable. Less than 0.75 per cent of gypsum is soluble in even 20 per cent phosphoric acid. The sulfate radical is one of the least toxic anions. The calcium ion exerts no appreciable toxic effect in concentrations less than one per cent. Since less than 0.6 per cent gypsum is soluble in 5 per cent phosphoric acid, it seems highly probable that the retarding effects of phosphates would be decidedly pronounced long before any slight depressive effect would occur from gypsum.

The effects of some inorganic cations used as stimulants

Waksman and Joffe (1922 b) and Joffe (1922 a) found no appreciable stimulation to sulfur oxidation from many organic and inorganic compounds tested. Joffe (1923 b) however, reported that some inorganic compounds greatly increased the velocity of sulfur oxidation. Hotchkiss (1923) observed stimulation to growth of *B. coli* from very small amounts of chlorides of Ce, Hg, Ni, Pb, Sn, Ti, and Zn. Investigations were conducted with some of the compounds which were apparently most stimulating in Joffe's experiments. It was of interest to know what effects stimulants would have on the sulfur-carbon metabolism of the organism and the intentions were to investigate this question if marked stimulation could be produced.

Compounds of zinc, caesium, nickel, lead, tin, and mercury were used. The concentrations in which these compounds were studied in the media are given in table 6 as p.p.m. of the elements and not of the compounds. Calculated amounts of stock solu-

TABLE 6

Stimulation to oxidation by some inorganic cations

CATION STUDIED	CATIONS FOUND STIMULATING BY		CATIONS USED	TITER*					
				After 6 days		After 10 days		After 14 days	
	Joffe	Hotchkiss		Per culture	After subtracting control	Per culture	After subtracting control	Per culture	After subtracting control
	p.p.m.	*p.p.m.*	*p.p.m.*						
0				5.36		10.79		12.89	
Zn (as $ZnSO_4 \cdot 6H_2O$)	10	0.65 to 3.27†	2	5.80	0.44	11.17	0.38	13.50	0.61
			10	4.77	−0.59	10.42	−0.37	13.03	0.14
Cs (as CsCl)	100	—	10	5.93	0.57	11.32	0.53	13.06	0.71
			50	4.27	−1.09	9.80	−0.99	11.63	−1.26
Ni (as $NiSO_4$)	Up to 400	0.3 to 5.88‡	5	5.17	−0.19	10.15	−0.64	12.22	−0.67
			25	2.80	−2.56	9.43	−1.36	12.60	−0.29
Hg (as $HgCl_2$)	—	0.22	0.05	3.27	−2.09	8.13	−2.66	10.33	−2.56
			0.25	0.07	−5.29	3.77	−7.02	10.17	−2.72
Pb (as $PbCO_3$)	10	9.3§	2	6.03	0.67	11.57	0.78	13.47	0.58
			10	5.67	0.31	11.13	0.34	13.37	0.48
Sn (as $SnCl_2$)	10	0.59 to 5.9¶	2	5.62	0.26	10.40	−0.39	12.50	−0.39
			10	5.70	0.34	11.77	0.98	14.17	1.28

* Averages of three flasks for each treatment. Titers of uninoculated media are substracted.
† As $ZnCl_2$.
‡ As $NiCl_2$.
§ As Pb Cl_2.
¶ As $SnCl_4$.

tions of these compounds were added to the sterilized sulfur media. The results in table 6 may indicate a significant stimulation from some of the cations, but this is not appreciable. Mercuric ions were distinctly toxic to oxidation in 0.05 p.p.m. and growth was temporarily inhibited at 0.25 p.p.m. The behavior of *Thiobacillus thiooxidans* seems different from that of *Bact. coli* since Hotchkiss noted stimulation to *Bact. coli* with 0.22 p.p.m. of mercury in the form of mercuric chloride. If any of the cations were stimulating this was the case with zinc, caesium, and lead compounds, in the low concentrations (2 p.p.m. for Zn and Pb, 10 p.p.m. for CS). Higher concentrations, (10 for Zn and Pb, 50 for CS) of these compounds, retarded oxidation. The higher concentrations were more toxic than the lower concentrations in every instance but one—in the case of 10 p.p.m. of tin as stannous chloride. This result is apparently erratic. One of three cultures receiving the same treatment was considerably more active than either of the other two. In another series the stimulating effect of the stannous chloride was not apparent.

No such stimulation to oxidation has been observed in these experiments as that reported by Joffe (1923 b). He indicated that toxic effects of nickel began only at 400 p.p.m. In these experiments, 25 p.p.m. distinctly depressed oxidation, particularly in the early stages of the process.

After incubation for fourteen days, cultures were transferred to fresh sterile media containing the same concentrations of the compounds as the initial cultures. No increases in oxidation over the initial cultures were noted in the transfers that could be ascribed to the cations used. The results obtained did not warrant further investigations in this direction.

Oxidation of some forms of sulfur differing in physical composition

Rhombic sulfur which has passed through a 60 mesh sieve has been used in practically all cases. In the experiments of table 7 sulfur was used in three different forms. The rhombic sulfur was similar to that usually added to the medium. The precipitated sulfur was prepared in the usual manner by precipitation from sulfides by mineral acids. This sulfur was very finely divided.

The "amorphous" sulfur undoubtedly contained some rhombic sulfur. In its preparation, sulfur was melted, rapidly cooled and ground to a fine powder after becoming brittle. This sulfur was probably the coarsest of the three but was all passed through a 60 mesh sieve. The sulfur was added in 1 gm. portions to the solution of the normal sulfur medium. The relative rates of oxidation are indicated in table 7.

TABLE 7

Oxidation of sulfur of different physical composition

PHYSICAL STATE OF THE SULFUR	TITER AFTER					
	6 days		11 days		16 days	
	Per culture	Average	Per culture	Average	Per culture	Average
Rhombic*	4.80	3.69	8.10	8.60	9.10	10.22
	3.05		6.45		7.40	
	3.50		8.30		9.80	
	4.10		8.75		10.20	
	3.00		11.40		14.60	
Precipitated†	2.95	2.99	8.70	8.78	12.65	13.81
	1.85		7.30		12.60	
	2.75		8.00		13.00	
	3.80		10.30		15.50	
	3.60		9.60		15.30	
Amorphous*	0.90	3.04	6.95	8.35	8.20	9.98
	3.90		10.20		11.40	
	2.50		6.80		8.70	
	3.70		9.30		11.50	
	4.20		8.50		10.10	

*Through a 60 mesh sieve.

† Passed through the finest sieve available—over 600 mesh.

All three forms of sulfur were rapidly oxidized and no very striking differences are apparent. It might be expected that the precipitated sulfur would become oxidized much more rapidly than either of the others due to its very fine state of division and the relatively greater surface exposed. No very great differences were observed in the early periods of incubation. Precipitated sulfur was more available after longer periods due largely to the

fact that it remained suspended on the surface of the medium much better than either of the other forms. It is recommended as a very desirable form of sulfur for use in the solution media for this reason.

Activity of cultures after long periods of incubation

An opinion has been held that the organism deteriorates after long periods of incubation in solution media. This opinion has been partly modified since the organism displayed no attenuated activity with prolonged cultivation by continuous transfer in the synthetic medium. The present experiment was designed to indicate whether cultures deteriorate after long periods of incubation. Cultures which had been incubated from seven to one hundred thirteen days were inoculated into fresh sterile media (table 8). There is no correlation between the activity of the bacterium and the period of incubation in the solution medium.

TABLE 8

Activity of cultures after long periods of incubation

AGE OF CULTURE USED FOR INOCULATION	TITER AFTER*	
	4 days	9 days
days		
113	3.33	10.95
107	2.70	9.15
98	3.10	8.70
91	2.80	11.43
85	2.70	8.83
81	2.70	11.08
71	2.18	12.00
67	2.20	10.35
33	2.10	9.43
26	2.73	11.60
7	4.08	10.80

* Averages of 2 flasks. Reported as increases over controls.

It has previously been pointed out that the decreasing rate of oxidation in aging cultures was probably caused by the accumulation of acid and was not due to any attenuation of the bacterium. This conclusion is further substantiated by the results of this experiment.

The gaseous environment

Unlike the denitrifying sulfur bacteria which can obtain oxygen from nitrates and carbon from dissolved carbonates, *Thiobacillus thiooxidans* requires an abundant supply of both oxygen and carbon dioxide. In the absence of either of these gases, growth ceases since only small amounts are soluble in the acid medium. In this respect the organism is similar to the itrifying bacteria. Winogradsky (1904–6, p. 166) states that " . . . . . keine Entwicklung des Nitritbildners und mithin keine Nitritation ohne freil bzw. halbgebundene Kohlensäure möglich ist." However, as Coleman (1908) and Bonazzi (1921) have indicated, once acid production by this organism is initiated, sufficient carbon dioxide is liberated from the carbonates to satisfy the carbon requirements without dependence upon atmospheric carbon dioxide. In the case of the nitrate formers, atmospheric carbon dioxide is indispensable even in the presence of carbonates since there is no reaction between the carbonates and the nitrate produced by oxidation. Atmospheric oxygen is, of course, required by both organisms.

TABLE 9

Oxidation under increased and reduced pressure

TREATMENT*	pH	TITER	INCREASE IN TITER OVER NORMAL ATMOSPHERE	PER CENT INCREASE IN TITER
Uninoculated	4.6	1.18		
Normal atmosphere	2.0	2.33		
	1.9	2.60		
Reduced pressure (90 mm.), 0.12 atmosphere	1.7	3.03	0.35	27.3
	1.9	2.60		
Increased pressure (46 mm.) 0.06 atmosphere	1.6	4.20	1.90	148.4
	1.5	4.53		

* Incubation period—4 days.

By increasing the partial pressure of carbon dioxide, the rapidity of oxidation of sulfur is hastened (Waksman and

Starkey, 1922). Apparently the amounts of carbon dioxide in the normal atmosphere are insufficient for the requirements of sulfur bacteria for maximum development. Slightly reduced pressure (0.12 atmosphere) has not appreciably affected the process. Slightly increased pressure (0.06 atmosphere) accelerated oxidation (table 9). This beneficial effect may be caused by the larger amounts of carbon dioxide dissolved in the media under increased pressure.

Temperature

The organism responds to high temperatures in the same manner as many other bacteria not producing spores; temperatures above 50°C. for even short intervals are fatal. A temperature of

TABLE 10

Oxidation of sulfur at different temperatures

TEMPERATURE	TITER AFTER				S AS SULFATE IN 100 CC. AFTER	
	2 days	4 days	6 days	8 days	3 days	8 days
°C.					mgm.	mgm.
10–13	0.34	1.15	1.65	2.04	11.81	56.04
22–23	1.41	4.01	6.61	8.15	72.25	237.62
26–27	2.56	5.46	7.91	10.68	112.90	312.06
35.5	2.98	5.35	6.35	7.20	132.96	211.03

55°C. for less than sixty minutes was sufficient to kill all of the cells of an active culture.

The following experiment was performed to disclose what effects temperature had on oxidation. After incubating for four days, cultures were filtered and divided into 100 cc. portions in 250 cc. flasks each containing 1 gram of sulfur. The initial cultures in all cases were therefore uniform. Four of these cultures were inoculated at each of four temperatures, 10 to 13°, 22 to 23°, 26 to 27° and 35.5°C. All cultures developed except two at 35.5°C. which died out. Initially, the other two cultures at this temperature developed very rapidly, but oxidation was retarded after incubating for four days. The reactions indicate that this is close to the temperature limiting growth. Only averages of the four cultures at each incubation temperature are recorded in

table 10. Cultures were not incubated at temperatures between 27° and 35°C. but maximum development would probably occur within this range. Lower temperatures retarded oxidation and the process was very slow at 10 to 13°C.

Response to desiccation

Joffe (1922a) noted that the sulfur bacterium survived short periods of drying in soils. However, on several occasions it has been observed that soils which previously harbored an active culture of *Thiobacillus thiooxidans* produced no growth when introduced into the sulfur medium after air-drying.

In this experiment, the duration of life of the organism in drying sulfur and soil was determined. Sulfur was filtered from pure cultures of the organism and spread out to dry in the incubator at 27°C. Periodically, flasks of the sterile sulfur medium were inoculated with portions of the sulfur and the moisture content was determined upon another sample of the sulfur. Soil which had been composted with sulfur for some time and was populated with an active culture of the organism was spread out in the incubator and treated in the same manner as the sulfur. Flasks of the sterile sulfur medium were inoculated from time to time with the soil and the moisture content of the soil was determined simultaneously.

Originally, both the soil and sulfur gave rapid oxidation when introduced into the sulfur medium. As indicated in table 11, the organisms survived for only a short time after desiccation commenced and the duration of life of the bacterium depended on the rates of desiccation. Sulfur dried out rapidly and contained only 0.5 per cent moisture after twenty-four hours at which time the sulfur organism had apparently died out since it produced no growth in the sulfur medium into which it was introduced. The soil dried out more slowly and the life of the organism persisted somewhat longer. Small numbers of active cells were present after two days at which time the moisture content had decreased to 2.3 per cent. After three days no active cells were apparently present since the organism failed to develop from the

soil in the sulfur medium. The moisture content was between 1.5 and 2 per cent.

Some of the desiccated sulfur was introduced into soil free from *Thiobacillus thiooxidans* and kept under favorable moisture and temperature conditions for several months. At intervals, portions of this soil were introduced into flasks of the sterile sulfur medium, but in no instance was growth observed.

It seems quite evident that the organism is not resistant to desiccation and that a comparatively dry environment is fatal in even a short period. Since there was no development of the

TABLE II

Response to dessiccation

DAYS OF DESSICATION	SULFUR			SOIL		
	Per cent moisture content	Growth in sulfur medium in 14 days	Titer of sulfur medium minus control	Per cent moisture content	Growth in sulfur medium in 14 days	Titer of sulfur medium minus control
0	58.7	+++	11.60	15.2	+++	8.75
¼	39.1	+++	8.90	12.2	+++	8.60
½	11.4	+++	17.50	9.4	+++	7.85
1	0.5	−	0	5.9	+++	7.60
2	0.3	−	0	2.3	+	5.35
3		−	0		−	0
4	0.4	−	0	1.4	−	0
5	0.3	−	0	1.3	−	0
7	0.4	−	0	1.1	−	0
11	0.5	−	0	1.1	−	0

organism in soil which should present favorable conditions for growth, it is further apparent that the organism died out and was not merely temporarily inactive in the desiccated material.

SUMMARY

Investigations have been conducted which were concerned with the physiology of the sulfur organism, *Thiobacillus thiooxidans* Waksman and Joffe. Some of the results may be summarized as follows:

1. Oxidation is most rapid in the early stages of the process, following a short lag period of about two days, and decreases as

the culture ages. The decreased rate of oxidation is not apparently due to any attenuation of the organism, or to the accumulation of any toxic organic metabolic products, but rather to the accumulation of sulfuric acid. When expressed graphically, the course of oxidation in solution cultures takes the form of a growth curve.

2. Growth was vigorous in the presence of 3 per cent sodium thiosulfate but 10 per cent of this salt inhibited oxidation. In the presence of high concentrations of thiosulfate, sulfur is precipitated in the medium during growth. The sulfur is probably precipitated indirectly and not as a product of the primary reaction of the process. With sodium thiosulfate as the source of energy, from 50 to 65 parts of sulfur in the form of thiosulfate are oxidized to sulfate per unit of carbon assimilated. The energy utilized for assimilation of carbon was only 4.3 to 5.4 per cent of the total energy available.

3. In the presence of 1 and 5 per cent sulfuric acid, the amounts of sulfur oxidized per unit of carbon assimilated were 36.6 and 43.0 respectively. The efficiency of the utilization of the energy available was lower under these conditions than in the normal medium.

4. The sulfur-carbon ratio is not appreciably affected by concentrations of primary potassium phosphate as high as 5.5 per cent. In the presence of 0.3, 1.4, and 5.5 per cent phosphate the ratios of sulfur oxidized to carbon assimilated were very much the same.

5. No appreciable stimulation to oxidation was observed in the presence of small amounts of salts such as zinc, caesium, nickel, mercury, lead, or tin. Slight stimulation may possibly be ascribed to salts of zinc, caesium, and lead.

6. Rhombic, precipitated, and amorphous sulfur are all rapidly oxidized. Precipitated sulfur is the most readily available of the three.

7. The organism does not deteriorate or become attenuated during long periods of incubation in synthetic inorganic solution media.

8. Growth ceases in the absence of either carbon dioxide or

oxygen. By increasing the partial pressure of carbon dioxide, the speed of oxidation is increased.

9. Slightly reduced pressure is ineffective on oxidation, but slightly increased pressure enhances the process.

10. Temperatures above 50°C. are fatal to the organism in a short time. Maximum oxidation occurs between 27° and 30°C. Oxidation is retarded at lower temperatures and is very slow at 10°C.

11. The organism is not resistant to desiccation and dies out readily in the absence of moisture.

REFERENCES

ALLISON, R. V. 1923 A preliminary note on the effect of the nitrate radical upon the biological oxidation of inorganic sulfur. N. J. Agr. Exp. Sta. Rpt. for 1922, p. 366–369.

BEIJERINCK, M. W. 1904a Plénoménes de reduction produits par les microbes. Archives des Sciences Exactes et Naturelles. Haarlem. Ser. II, **9**, 131–157. Also in Verzamelde Geschriften van M. W. Beijerinck, Bruckmann & Obernetter, München, **4**, 1921, p. 192–211.

BEIJERINCK, M.W. 1904b Ueber die Bakterien welche sich im Dunkeln mit Kohlensäure als Kohlenstoffquelle ernähren können. Centralbl. Bakt. (etc.), Abt. 2, **11**, 592–599. Also in Verzamelde Geschriften van M. W. Beijerinck, Bruckmann and Obernetter, München, **4**, 1921, p. 242–248.

BONAZZI, A. 1921 On nitrification. IV. The carbon and nitrogen relations of the nitrite ferment. Jour. Bact., **6**, 479–499.

BROWN, H. D. 1923 Sulfofication in pure and mixed cultures with special reference to sulfate production, hydrogen-ion concentration, and nitrification. Jour. Amer. Soc. Agron., **15**, 350–382.

COLEMAN, L. C. 1908 Untersuchungen über Nitrifikation. Centralbl. Bakt. (etc.), Abt. 2, **20**, 401–420, 484–513.

DÜGGELI, M. 1919 Die Schwefelbakterien. Neujahrsblatt d. Naturforschenden Gesellschaft in Zürich. No. 121, 0.1–44.

FALK, I. S. 1923 Bibliographic review. The rôle of certain ions in bacterial physiology. A review (Studies on salt action, VII). Abs. Bact., **7**, 35.

GEHRING, A. 1915 Beiträge zur Kenntnis der Physiologie und Verbreitung denitrifizierenden Thiosulfat Bakterien. Centralbl. Bakt. (etc.), Abt. 2, **42**, 402–438.

HOTCHKISS, M. 1923 Studies on salt action. VI. The stimulating and inhibitive effect of certain cations upon bacterial growth. Jour. Bact., **8**, 141–162.

JACOBSEN, H. C. 1912 Die Oxydation von elementarem Schwefel durch Bakterien. Folia Microbiologica, **1**, 487–496.

JACOBSEN, H. C. 1914 Die Oxydation von Schwefelwasserstoff durch Bakterien. Folia Microbiologica, **3**, 155–162.

JOFFE, J. S. 1922a Biochemical oxidation of sulfur and its significance to agriculture. N. J. Agr. Exp. Sta. Bul. 374, 91 p.

JOFFE, J. S. 1922b Preliminary studies on the isolation of sulfur-oxidizing bacteria from sulfur-floats-soil composts. Soil Sci., **13**, 161–172.

JOFFE, J. S. 1922c Studies of sulfur oxidation in sulfur-floats-soil mixtures. Soil Sci., **13**, 107–118.

JOFFE, J. S. 1923a Acid phosphate by the biological process. N. J. Agr. Exp. Sta. Rpt. for 1922, p. 361–365.

JOFFE, J. S. 1923b Homogeneous catalysts in the oxidation of sulfur by *Thiobacillus thiooxidans*. Abs. Bact., **7**, 8.

KLEIN, G., AND LIMBERGER, A. 1923 Zum Kreislauf des Schwefels im Boden. (Ein Beitrag zur Biologie der Thiosulfatbakterien.) Biochem. Zeit., **340**, 473–483.

LIESKE, R. 1912 Untersuchungen über die Physiologie denitrifizierender Schwefelbakterien. Ber. Deut. Botan. Gesell., **30**, 12–22.

LIPMAN, J. G. 1923 Recent investigations on the oxidation of sulfur by microörganisms. Jour. Indus. Engin. Chem., **15**, 404–405.

LIPMAN, J. G., WAKSMAN, S. A., AND JOFFE, J. S. 1921 The oxidation of sulfur by soil microörganisms. I. Soil Sci., **12**, 475–489.

NATHANSOHN, A. 1902 Ueber eine neue Gruppe von Schwefelbakterien und ihren Stoffwechsel. Mitt. a. d. zool. Station Neapel, **15**, 655. Cf. Centralbl. Bakt. (etc.), Abt. 2, 1904, **11**, 109.

TRAUTWEIN, K. 1921 Beitrag zur Physiologie und Morphologie der Thionsäurebakterien. (Omelianski). Centralbl. Bakt. (etc.), Abt. 2, **53**, 513–548.

TRAUTWEIN, K. 1924 Die Physiologie und Morphologie der fakultativ autotrophen Thionsäurebakterien unter heterotrophen Ernährungsbedingungen. Centralbl. Bakt. (etc.), Abt. 2, **61**, 1–5.

WAKSMAN, S. A. 1922a Microörganisms concerned in the oxidation of sulfur in the soil. I. Introduction. Jour. Bact., **7**, 231–238.

WAKSMAN, S. A. 1922b Microörganisms concerned in the oxidation of sulfur in the soil. III. Media used for the isolation of sulfur bacteria from the soil. Soil Sci., **13**, 329–336.

WAKSMAN, S. A. 1922c Microörganisms concerned in the oxidation of sulfur in the soil. IV. A solid medium for the isolation and cultivation of *Thiobacillus thiooxidans*. Jour. Bact., **7**, 605–608.

WAKSMAN, S. A. 1922d Microörganisms concerned in the oxidation of sulfur in the soil. V. Bacteria oxidizing sulfur under acid and alkaline conditions. Jour. Bact., **7**, 609–616.

WAKSMAN, S. A., AND JOFFE, J. S. 1921a Acid production by a new sulfur-oxidizing bacterium. Science, n.s., **53**, 216.

WAKSMAN, S. A., AND JOFFE, J. S. 1921b The oxidation of sulfur by microörganisms. Proc. Soc. Exper. Biol. and Med., **18**, 1–3.

WAKSMAN, S. A., AND JOFFE, J. S. 1922a The chemistry of the oxidation of sulfur by microörganisms to sulfuric acid and transformation of insoluble phosphates into soluble forms. Jour. Biol. Chem., **50**, 35–45.

WAKSMAN, S. A., AND JOFFE, J. S. 1922b Microörganisms concerned in the oxidation of sulfur in the soil. II. *Thiobacillus thiooxidans*, a new sulfur-oxidizing organism isolated from the soil. Jour. Bact., **7**, 239–256.

WAKSMAN, S. A., AND STARKEY, R. L. 1922 Carbon assimilation and respiration of autotrophic bacteria. Proc. Soc. Exper. Biol. and Med., **20**, 9–14.

WAKSMAN, S. A., AND STARKEY, R. L. 1923 On the growth and respiration of autotrophic bacteria. Jour. Gen. Physiol., **5**, 285–310.

WAKSMAN, S. A., WARK, CLARA H., JOFFE, J. S., AND STARKEY, R. L. 1923 Oxidation of sulfur by microörganisms in black alkali soils. Jour. Agr. Res., **24**, 297–305.

WINOGRADSKY, S. 1904–6 Nitrification. Handbuch der Technischen Mykologie (Lafar), v. 3. Mykologie des Bodens, des Wassers, und des Düngers. Part 1, chap. 2, p. 132–192. Fischer, Jena.

CONCERNING THE CARBON AND NITROGEN NUTRITION OF THIOBACILLUS THIOOXIDANS, AN AUTOTROPHIC BACTERIUM OXIDIZING SULFUR UNDER ACID CONDITIONS[1]

ROBERT L. STARKEY

From the Department of Soil Chemistry and Bacteriology, New Jersey Agricultural Experiment Stations, New Brunswick, New Jersey

Received for publication July 21, 1924

INTRODUCTION

In the course of recent investigations the identity of *Thiobacillus thiooxidans*, Waksman and Joffe, the agent which is responsible for the formation of acid in sulfur-rock phosphate-soil composts has been established and the isolation of this agent has been accomplished. Satisfactory methods of cultivation have been devised and a number of the physiological characteristics of the organism have been determined. It has been demonstrated that the organism is autotrophic, that sulfur is rapidly oxidized, and that considerable quantities of sulfuric acid accumulate.

By reason of its unusual physiology, the bacterium is outstanding. Due to the simplicity of the basic reactions involved in its nutrition the organism readily lends itself to experimentation. The nature of the end product of oxidation is such that this biological process may find some practical applications.

The utilization of organic compounds and inorganic nitrogenous compounds by this bacterium are still somewhat obscure. It is the purpose of this contribution to consider some effects which these two kinds of compounds may have on the metabolism of the organism. References to reviews and previous inves-

[1] Paper No. 190 of the Journal Series of the New Jersey Agricultural Experiment Stations, Department of Soil Chemistry and Bacteriology.

tigations concerned with the biological oxidation of sulfur and inorganic sulfur compounds may be obtained from a recent contribution (Starkey, 1925).

EXPERIMENTAL METHODS

The cultural and analytical methods followed in this work were, in general, the same as outlined in preceding papers (Waksman and Starkey, 1923; Starkey, 1925). A few other methods of analysis have been used in the following experiments.

Nitrates were determined by the colorimetric phenoldisulfonic acid method. The culture solutions were neutralized with sodium hydroxide before evaporation.

Glucose was determined by Bertrand's method (1906). The glucose solution was boiled with alkaline copper sulfate solution and the precipitated cuprous oxide filtered on filter paper. The copper oxide was dissolved in ferric sulfate and the resultant ferrous sulfate titrated with standard potassium permanganate.

CARBON NUTRITION

It has been quite definitely determined (Waksman and Starkey, 1922, 1923; Starkey, 1925) that the organism makes no growth in the absence of carbon dioxide and that organic carbon cannot replace carbon dioxide as the source of carbon. In the absence of sulfur or inorganic sulfur compounds as sources of energy there is no growth. Organic compounds included in media in the presence of inorganic sources of energy may undergo some changes which are not apparent when they are present as the sole sources of energy. Waksman and Joffe (1922b) found that glycerol, alcohol, mannitol, and glucose favored oxidation, acting either as stimulants or playing a part in the structural requirements of the organism. Joffe (1922) mentioned that pepton inhibited oxidation, but the amounts used and the extent of the effects produced were not given. It was also stated by Joffe (1922) that carbohydrates or other organic sources seem to be used by the bacterium. Up to the present time, however, data have not been presented sufficient to indicate that *Thiobacillus*

thiooxidans can utilize any organic compounds incorporated with the sulfur medium.

Glucose and oxidation of sulfur

Winogradsky and Omeliansky (1899) found that glucose definitely injured growth of nitrate formers at 0.0025 molar or 0.045 per cent and completely inhibited growth at 0.015 molar or 0.27 per cent concentration. The nitrate formers were more sensitive to injury than the nitrite formers. Injury to respira-

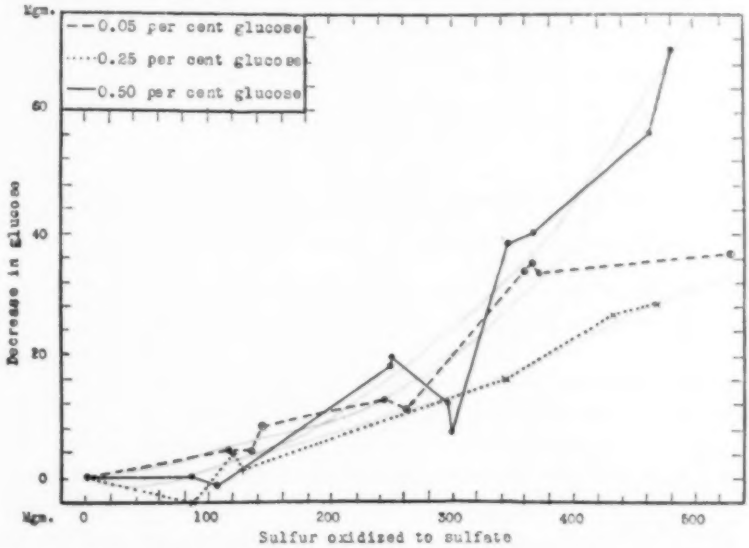


FIG. 1. CORRELATION BETWEEN DISAPPEARANCE OF GLUCOSE AND INCREASE IN SULFATE IN SOLUTION CULTURES

tion of the nitrate formers was only 25 to 30 per cent at 0.8 molar concentration as determined by Meyerhoff (1916 a and b) For the nitrite formers injury to respiration was 40 per cent at 0.6 molar concentration. Coleman (1908) noted no injury to growth of nitrate formers at 0.02 to 0.05 per cent glucose in sand or solution cultures, but larger amounts of glucose were distinctly toxic. No nitrite formation took place at 0.2 per cent glucose and even 0.02 per cent was found injurious to nitrite formers.

TABLE 1

Disappearance of glucose in solution cultures

DAYS OF INCUBATION	CONTROL*	TITER		S AS SULFATE IN 100 CC.		GLUCOSE IN 100 CC.			
		Per culture	After subtracting control	Per culture	After subtracting control	1	2	Average	Decrease from blank
				mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
5	+	1.08		18.74		0	0	0	
5	−	4.35	3.27	124.29	105.55	0	0	0	0
5	−	4.70	3.62	69.36	50.62	0	0	0	0
10	+	1.08		18.54		0	0	0	
10	−	9.28	8.20	284.99	266.45	0	0	0	0
10	−	9.03	7.95	278.12	259.58	0	0	0	0
21	+	1.13		18.20		0	0	0	
21	−	15.10	13.97	481.03	462.83	0	0	0	0
21	−	12.80	11.67	400.35	382.15	0	0	0	0
5	+	1.05		17.51		41.61	50.06	45.84	
5	−	5.18	4.13	153.48	135.97	41.61	41.61	41.61	4.23
5	−	4.55	3.50	137.07	119.56	41.61	41.61	41.61	4.23
8	+	1.08		18.20		41.75	44.25	43.00	
8	−	5.50	3.42	162.20	144.00	32.75	36.50	34.63	8.37
8	−	9.15	8.07	281.55	263.35	26.50	29.00	27.55	15.25
10	+	1.05		17.51		41.61	43.00	42.31	
10	−	8.50	7.45	261.50	243.99	25.37	33.37	29.37	12.94
16	+	1.08		18.20		41.75	44.25	43.00	
16	−	16.40	15.32	383.87	365.67	10.70	9.50	10.10	32.90
20	−	14.25	13.17	379.40	361.20	13.10	13.10	13.10	29.90
21	+	1.10		18.20		48.67	50.06	49.37	
21	−	17.20	16.10	546.96	528.76	12.40	12.40	12.40	36.97
21	−	12.55	11.45	390.39	372.19	16.25	15.00	15.63	33.74
5	+	1.03		16.48		241.95	241.95	241.95	
5	−	2.63	1.60	103.90	87.42	246.10	246.10	246.10	−4.15
5	−	4.83	3.80	143.73	127.25	236.40	250.30	243.35	1.40
10	+	1.00		17.17		239.15	241.95	240.55	
10	−	4.63	3.60	134.59	117.42	236.25	236.25	236.25	4.30
10	−	11.50	10.50	361.21	344.04	226.40	222.05	224.23	16.32
21	+	1.08		16.48		241.95	231.95	236.95	
21	−	14.28	13.20	448.41	431.93	210.85	209.45	210.15	26.80
21	−	15.30	14.22	484.48	468.00	215.00	202.50	208.75	28.20
5	+	0.98		16.62		478.25	488.00	483.13	
5	−	3.50	2.52	101.63	85.01	482.80	482.80	482.80	0.33
5	−	4.15	3.17	122.92	106.30	484.40		484.40	−1.27

TABLE 1—*Continued*

DAYS OF INCUBATION	CONTROL*	TITER		S AS SULFATE IN 100 CC.		GLUCOSE IN 100 CC.			
		Per culture	After subtracting control	Per culture	After subtracting control	1	2	Average	Decrease from blank
				mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
8	+	1.05		17.30		488.00	489.65	488.83	
8	−	8.70	7.65	266.44	249.14	470.65	467.50	469.08	19.75
8	−	8.70	7.65	265.62	248.32	472.20	469.05	470.63	18.20
10	+	0.98		16.16		490.95	488.00	489.48	
10	−	10.13	9.15	315.34	299.18	479.70	484.40	482.05	7.43
10	−	10.05	9.07	313.96	297.80	473.15	481.25	477.20	12.28
16	+	1.05		17.30		488.00	489.65	488.83	
16	−	12.28	11.23	362.83	345.53	454.05	446.25	450.15	38.68
20	−	14.30	13.25	383.87	366.57	444.70	452.50	448.60	40.23
21	+	0.98		16.62		478.25	488.00	483.13	
21	−	15.85	14.87	496.14	479.98	416.80	422.00	419.40	70.08
21	−	15.15	14.17	476.58	460.42	435.56	430.00	432.78	56.70

* + indicates uninoculated controls; − indicates inoculated cultures.

Apparently glucose is quite injurious to the growth of nitrifying bacteria even though respiration for short intervals is not as injuriously affected.

Oxidation by *Thiobacillus thiooxidans* was not appreciably affected in concentrations as high as 5 per cent glucose (Waksman and Starkey, 1923). It was practically inhibited in 10 per cent glucose solution. Growth of this organism is not as sensitive to glucose as is growth of nitrifying bacteria. There even appeared to be increased oxidation in the presence of 1 and 5 per cent glucose.

In order to ascertain whether there was any consumption of glucose by the organism several experiments were performed. Media were prepared containing 0.05, 0.25, and 0.50 per cent glucose in addition to the contents of the normal sulfur medium. The glucose was sterilized separately in standard solution and added to the sterile sulfur medium. Periodically, cultures were analyzed for acidity, sulfates, and glucose. Some of the data are presented in table 1 and figure 1.

Without doubt, glucose disappears from the medium during

incubation. Although there is considerable variability between duplicate determinations of glucose, particularly in the presence of large amounts, the extent of the decreases in glucose is significant. As growth and oxidation proceed, larger amounts of glucose disappear. There are, in general, greater losses of glucose in the presence of greater accumulations of acid which indicate advanced growth and oxidation. This gives a general but not a consistent proportion between the amounts of sulfur oxidized and glucose which has disappeared. There is, however, no close correlation between the amounts of glucose initially present in the medium and the amounts which disappear during the oxidation process. These facts are apparent from the figure. There was definite disappearance of glucose when present in amounts of 0.05, 0.25. and 0.50 per cent. Per unit of sulfur oxidized, more glucose disappeared at 0.05 than at 0.25 per cent. However, at 0.50. per cent, more glucose disappeared per unit of sulfur oxidized than at either of the other concentrations, in some instances, while in others the reverse was true.

Coleman (1908) also reported definite disappearance of glucose in cultures of nitrifying bacteria. His results led him to conclude that ". . . . Von allen diesen Möglichkeiten scheint mir diejenige des Dextrose verbrauchs als Ersatzenergiequelle am wahrscheinlichsten."

Conditions are somewhat different in cultures of this sulfur organism from those of nitrifying bacteria. In the latter case the medium is alkaline; in cultures of the sulfur bacterium there is a progressive and appreciable increase in acidity. In order to discover whether the acid itself effected hydrolysis of the glucose the following experiment was conducted. Portions of the sulfur medium were prepared, as in the preceding experiment, containing 0.05 and 0.25 per cent glucose. Some of the media was left unchanged. To the rest, sulfuric acid was added to bring the media to acidities commonly produced by the oxidation of sulfur. Two concentrations of the acid were created, one of about 0.1 N acid and the other about 0.3 N acid. These media were left uninoculated and kept at the normal incubation temperature. Periodically, determinations were made for glucose and for acid-

ity by titration. The results are presented in table 2. There is no appreciable decrease in glucose in any of the uninoculated

TABLE 2

Stability of glucose in uninoculated acid media

DAYS OF INCUBATION	0.5 N H_2SO_4 IN 100 CC. OF MEDIUM	TITER	GLUCOSE IN 100 CC.			
			1	2	Average	Decrease from control
	cc.		*mgm.*	*mgm.*	*mgm.*	*mgm.*
7	0	1.10	43.17	46.00	44.59	
7	20	5.70	50.24	50.24	50.24	−5.65
7	20	5.80	47.39	53.12	50.26	−5.67
7	60	15.15	50.24	48.78	49.51	−4.92
7	60	14.90	48.78	50.24	49.51	−4.92
15	0	1.10	40.33	41.72	41.03	
15	20	5.75	53.12	50.24	51.68	−10.65
15	20	5.95	48.78	56.00	52.39	−11.36
15	60	15.20	48.78	53.12	50.95	−9.92
15	60	15.20	44.59	53.12	48.86	−7.83
21	0	1.10	50.24	47.39	48.82	
21	20	6.05	44.59	51.66	48.13	0.69
21	20	5.95	54.55	53.12	53.84	−5.02
21	60	15.30	58.89	56.00	57.45	−8.63
21	60	15.10	50.24	53.12	51.68	−2.86
7	0	1.05	238.30	235.55	236.93	
7	20	5.90	258.55	239.45	249.00	−12.07
7	20	5.70	259.70	259.70	259.70	−22.77
7	60	14.95	251.20	255.55	253.38	−16.45
7	60	14.85	245.30	254.10	249.70	−12.77
15	0	1.00	236.95	245.30	241.13	
15	20	5.75	249.70	264.10	256.90	−15.77
15	60	15.20	264.10	251.20	257.65	−16.52
21	0	1.05	245.30	251.20	248.25	
21	20	5.70	247.20	248.30	247.75	0.50
21	20	5.80	251.20	256.95	254.08	−5.83
21	60	15.65	251.20	256.95	254.08	−5.83
21	60	15.40	249.70	248.30	249.00	−0.75
21*	20	16.55	222.95	220.00	221.48	26.77
21*	60	25.55	204.45	211.40	207.93	40.32

* Inoculated.

media even after considerable periods of time. This seems to give ample evidence that the acid produced by the oxidation of sulfur was not alone responsible for the disappearance of glucose

in the cultures. Two flasks of media (the last two reported in the table) were inoculated. There was a marked disappearance of glucose in both instances. In the presence of acid, the results of the determinations for glucose ran consistently higher than in its absence. This may be the factor responsible for some of the erratic results obtained with the cultures in the preceding experiment where some of the glucose determinations were apparently higher in the inoculated cultures than in the controls. The effects of the acid would tend to conceal any slight disappearance of glucose and the actual amounts would be even greater than the calculated results presented in table 1.

For another experiment, media were prepared like the normal sulfur medium omitting the sulfur and including glucose in amounts of 0.05, 0.25, and 0.50 per cent. These media were inoculated and incubated as usual. As would naturally be predicted from some earlier experiments (Waksman and Starkey, 1922 and 1923) no growth took place in any of these media (table 3). There are no variations in the glucose contents of these media that cannot be interpreted as within the error of the method of analysis. This experiment again indicates that no growth takes place in the absence of the inorganic sources of energy.

In a further experiment, certain quantities of glucose were added to cultures which were analyzed for glucose after an interval of a few hours. There were negligible differences between these results and determinations for the same amounts of glucose in distilled water. The bacterial cells apparently did not interfere with the determination for glucose.

These experiments suggested the possibility that the glucose might have been incidentally hydrolyzed in the cultures outside of the cells by some metabolic products excreted by the organism. In order to throw light upon this question the following experiment was conducted. Several cultures of the organism were filtered free from bacterial cells and suspended matter by passage through a Berkefeld filter. This filtrate was divided into three portions under aseptic conditions. One portion was unaltered. Amounts of sterile standard solutions of glucose were

TABLE 3

Stability of glucose in inoculated media lacking sulfur

DAYS OF INCUBATION	CONTROL*	TITER		GLUCOSE IN 100 CC.			
		Per culture	After subtracting control	1	2	Average	Decrease from blank
				mgm.	*mgm.*	*mgm.*	*mgm.*
7	+	1.05		41.72	41.72	41.72	
7	−	1.05	0	46.00	44.59	45.30	−3.58
7	−	1.05	0	40.33	40.33	40.33	1.39
15	+	1.10		44.59	44.59	44.59	
15	−	1.05	−0.05	46.00	50.24	48.12	−3.53
21	+	1.10		48.77	48.77	48.77	
21	−	1.05	−0.05	53.12	53.12	53.12	−4.35
21	−	1.10	0	43.17	53.12	48.15	0.62
7	+	1.00		228.60	227.20	227.90	
7	−	1.00	0	223.55	228.60	226.08	1.82
7	−	1.00	0	232.75	221.45	227.10	0.80
15	+	1.00		231.40	245.30	238.35	
15	−	1.05	0.05	242.50	227.20	234.85	3.50
21	+	1.05		246.65	239.70	243.18	
21	−	1.10	0.05	249.70	236.95	243.33	−0.15
21	−	1.10	0.05	246.65	254.10	250.38	−7.20
7	+	1.00		460.00	451.70	455.85	
7	−	0.95	−0.05	457.20	457.20	457.20	−1.35
7	−	0.95	−0.05	434.40	457.20	445.80	10.05
21	+	1.05		513.90	508.20	511.05	
21	−	1.00	−0.05	511.10	505.30	508.20	2.85

* + indicates uninoculated media; − indicates inoculated media.

TABLE 4

Stability of glucose in Berkefeld filtrate from fourteen day old cultures of Thiobacillus thiooxidans

PERIOD OF ACTION OF FILTRATE	GLUCOSE IN 100 CC.			
	1	2	Average	Increase or decrease from control
hours	*mgm.*	*mgm.*	*mgm.*	*mgm.*
0	0	0	0	
	52.29	58.00	55.15	
	295.76	300.72	298.24	
4	59.12	59.12	59.12	+3.97
	290.76	292.00	291.38	−6.86
14	0	0	0	0
	56.89	54.61	55.75	+0.60
	303.04	305.48	304.26	+6.02

added to the other two portions in order to produce approximately 0.05 and 0.25 per cent glucose. Determinations for glucose were made immediately in all three solutions and again after incubation at 27°C. for four and for fourteen hours. The results appear in table 4. Oxidation by the cultures had produced approximately 0.15 N acid at the time of filtering and adding the glucose.

The results presented in the table fail to indicate significant differences in the amounts of glucose found in the filtrates at the different periods. From this experiment it can hardly be concluded that glucose disappears in a cultural filtrate of the organism in the absence of developing cells.

From the preceding experiments it has been indicated that glucose disappears during normal active oxidation in sulfur media. It has not been hydrolyzed by acid under the same conditions as existed in the cultures without the presence of developing bacteria. A culture of active cells did not interfere with the determination of glucose. In many instances the cultures have been plated on solid media commonly used in this laboratory for cultivation of fungi and bacteria. On such plates there was no development in any instance except an occasional contaminating organism introduced from the laboratory air during plating.

The assimilation of carbon by the organism in the presence of a small amount of glucose (0.066 per cent) has been previously reported (Waksman and Starkey, 1923). The average sulfur-carbon ratio was 39.22, a somewhat higher ratio than for the metabolism of the organism in the absence of glucose (31.75). In the light of the experiments just reported, the explanation of the higher ratio may be found in the disappearance of the glucose. Any decrease in the carbon added to the medium as glucose would tend to increase the ratio. It has not been discovered, however, whether the carbon is removed from the medium or exists there as a transformed product. In the latter instance there should be no effect on the sulfur-carbon ratio. It has been shown that even 5 per cent glucose is not injurious to the organism and the small amount of 0.066 per cent should exert no harmful effect. Consequently, a normal sulfur-carbon ratio would be predicted. The evidence at hand strongly suggests

that glucose in small amounts may enter into the metabolism of the cells in the presence of sulfur as a source of energy and may be used either as a source of energy or as carbon for growth, or both.

Oxidation in the presence of certain concentrations of citric acid

Since glucose was not found to be injurious even at comparatively high concentrations, it was of interest to discover whether an organic acid would be equally ineffective. From the results in table 5 it is evident that citric acid exerts no appreciable injurious effect at a concentration of 1 per cent. Oxidation was initi-

TABLE 5

*Concentration of citric acid and oxidation**

PER CENT CONCENTRATION	TITER AFTER		S AS SULFATE IN 100 CC. AFTER	
	3 days	15 days	3 days	15 days
			mgm.	*mgm.*
0	1.55	12.30	44.22	355.43
0.01	0.00	8.50	0.00	219.88
0.10	0.75	6.30	30.22	207.04
1.00	1.45	10.80	52.19	349.05
2.50	0.00	6.85	0.00	228.74
5.00	0.00	0.00	0.00	0.00
10.00	0.00	0.00	0.00	0.00
20.00	0.00	0.00	0.00	0.00

* Data are given as averages of duplicates for each treatment minus the uninoculated control media.

ally delayed at 2.5 per cent but was rapid after the first few days. At 5 per cent and above no oxidation took place. The injury was undoubtedly due to the organic radical and not to the acidity produced at these high concentrations.

Some amino acids and alcohols were found to be injurious at considerably lower concentrations (Waksman and Starkey, 1922).

NITROGEN NUTRITION

The nitrogen nutrition of the organism has been differently estimated in various reports. From experiments with crude composts, McLean (1918) concluded, "The addition of 0.2 per

cent of sodium nitrate was found to inhibit sulfofication processes almost entirely." From some of the first pure culture studies the statement was made (Lipman, Waksman and Joffe, 1921) that "As nitrogen sources, it can use ammonium salts and nitrates, but since the first tend to leave the medium more acid than the second, the first are preferred." Later Waksman and Joffe, 1922 a), "The nitrogen can be supplied in the form of inorganic or organic materials." Again (1922 b), "The best sources of nitrogen are ammonium salts of inorganic acids (particularly sulfate) followed by the ammonium salts of organic acids, after which come the nitrates, asparagin, and amino acids. Nitrites, in concentrations used (2 grams per liter) are toxic. Good growth is obtained with peptone, but the amount of sulfur oxidized is less than with the other sources of nitrogen."

From observations on the oxidation of sulfur in soils, Allison (1923) concluded that "sodium nitrate in any considerable quantity severely retarded and at times completely inhibited" oxidation of sulfur. From pure culture studies in solution media the same author believed oxidation was greater in the presence of nitrate than of ammonium sulfate. Joffe (1922) concluded that organic sources of nitrogen are readily used, that ammonium salts (especially sulfates) are not the best sources of nitrogen, that potassium nitrate is about the best source of nitrogen, and that sodium nitrate is not injurious to the organism.

The following experiments were conducted with the object of determining what sources of nitrogen are available to the organism with particular reference to nitrate and ammonium nitrogen.

Availability of nitrate and ammonium nitrogen

A medium was prepared containing no nitrogen other than what might be included in the c.p. chemicals used. To other media, potassium nitrate was added in amounts of 0.05, 0.25, and 1.25 per cent. In another series of media, 0.04 per cent ammonium sulfate was included in the absence of nitrate and in the presence of 0.05, 0.25, and 1.25 per cent potassium nitrate. Six cultures were used for each treatment in order to eliminate errors from variability in the cultures. The data are presented

TABLE 6

Availability of nitrate and ammonium nitrogen

PER CENT OF KNO_3 ADDED	CONTROL*	pH, AFTER			TITER, AFTER					
					5 days		10 days		15 days	
		5 days	10 days	15 days	Per culture	After subtracting control	Per culture	After subtracting control	Per culture	After subtracting control
0	+	4.6	4.6	4.6	1.35		1.30		1.40	
	−	2.0	1.2	−1.2	3.30		4.25		5.30	
	−	2.0	1.2	−1.2	3.20		4.30		5.50	
	−	2.0	1.2	−1.2	3.10	1.85	4.45	3.10	5.60	4.12
	−	2.0	1.2	−1.2	3.30		5.00		6.20	
	−	2.0	1.2	−1.2	3.10		4.20		5.25	
	−	2.0	1.2	−1.2	3.20		4.20		5.25	
0.05	+	4.6	4.6	4.6	1.30		1.30		1.40	
	−	2.0	1.4	−1.2	3.00		3.90		4.80	
	−	2.0	1.4	−1.2	2.80		3.80		4.45	
	−	2.0	1.4	−1.2	3.10	1.68	3.90	2.67	4.70	3.40
	−	2.0	1.4	−1.2	3.20		4.20		4.95	
	−	2.0	1.6	−1.2	2.70		3.60		4.40	
	−	2.0	1.2	−1.2	3.10		4.40		5.50	
0.25	+	4.6	4.6	4.6	1.35		1.35		1.35	
	−	2.0	1.4	−1.2	3.30		4.10		4.80	
	−	2.0	1.4	−1.2	3.00		3.90		4.65	
	−	2.2	1.6	−1.2	2.70	1.26	3.50	2.15	4.20	2.93
	−	2.2	1.4	−1.2	2.45		3.60		4.50	
	−	2.2	1.6	−1.2	2.60		3.50		4.30	
	−	3.2	1.8	1.6	1.60		2.40		3.20	
1.25	+	4.6	4.6	4.6	1.30		1.30		1.35	
	−	4.6	4.6	4.2	1.30		1.30		1.35	
	−	4.6	4.6	4.2	1.35		1.35		1.35	
	−	4.0	4.0	4.2	1.30	0.01	1.30	0.03	1.35	0.03
	−	4.4	4.0	4.2	1.30		1.30		1.40	
	−	4.6	4.4	4.4	1.30		1.30		1.40	
	−	4.4	4.0	4.2	1.30		1.40		1.40	
0.04 $(NH_4)_2SO_4$	+	4.6			1.30		1.30		1.35	
	−	−1.2			6.30		11.90		13.20	
	−	−1.2			7.80		12.95		14.80	
	−	2.6	−1.2		2.10	3.90	10.00	9.32	12.60	11.09
0.04	−	2.0	−1.2		2.75		8.95		11.45	
	−	−1.2			5.90		9.30		10.60	
	−	−1.2			6.35		10.60		12.00	

TABLE 6—*Continued*

PER CENT OF KNO_3 ADDED	CONTROL*	pH, AFTER			TITER, AFTER					
					5 days		10 days		15 days	
		5 days	10 days	15 days	Per culture	After subtracting control	Per culture	After subtracting control	Per culture	After subtracting control
0.04 $(NH_4)_2SO_4$ + 0.05 KNO_3	+	4.6			1.40		1.35		1.40	
	−	−1.2			6.70		9.70		10.70	
	−	−1.2			7.10		11.25		12.20	
	−	3.2	−1.2		1.50	4.60	9.30	8.78	11.20	9.85
	−	−1.2			6.50		9.80		10.25	
	−	−1.2			6.40		9.80		10.95	
	−	−1.2			7.80		10.90		12.20	
0.04 $(NH_4)_2SO_4$ + 0.25 KNO_3	+	4.6			1.35		1.40		1.40	
	−	−1.2			6.40		8.40		9.00	
	−	−1.2			6.10		8.50		9.20	
	−	−1.2			5.60	4.27	8.15	7.21	9.10	8.17
	−	−1.2			7.50		10.00		10.90	
	−	3.0			1.70		8.00		9.90	
	−	−1.2			6.40		8.60		9.30	
0.04 $(NH_4)_2SO_4$ + 1.25 KNO_3	+	4.6	4.6	4.6	1.30		1.35		1.40	
	−	4.0	4.0	4.0	1.35		1.40		1.40	
	−	4.0	4.0	4.0	1.35		1.35		1.40	
	−	4.6	4.6	4.2	1.40	0.04	1.40	0.03	1.35	−0.01
	−	4.0	4.0	4.0	1.30		1.40		1.40	
	−	4.2	4.0	4.2	1.30		1.40		1.40	
	−	4.2	4.6	4.6	1.35		1.35		1.40	

* + control uninoculated media; − inoculated media.

in table 6. The determinations on all of the cultures are presented in order to indicate the variability in oxidation commonly occurring between different cultures similarly treated. In the absence of ammonium nitrogen oxidation was very feeble and the lack of the turbidity which is characteristic of normal growth was very striking. However, there was more rapid oxidation where no nitrogen was added than where potassium nitrate was present even in a concentration of 0.05 per cent. The source of nitrogen for such oxidation was undoubtedly ammonium compounds contained as impurity in the compounds incorporated in the sulfur

medium or ammonia which might have been absorbed from the air during the period of incubation. The amounts of nitrogen necessary for small amounts of growth are naturally very slight.

There was no greater oxidation in any instance in the presence of nitrate which would indicate that nitrate was not used as a source of nitrogen. Conversely, in practically every culture containing nitrate, oxidation has been depressed over cultures similarly treated but containing no nitrate.

In the presence of ammonium nitrogen, added as the only source of nitrogen, oxidation was normal. Oxidation was, however, depressed in 0.05 per cent potassium nitrate and in all cases oxidation was completely inhibited in the presence of 1.25 per cent potassium nitrate. The concentration which completely inhibits growth is about 1 per cent potassium nitrate (Waksman and Starkey, 1922, 1923). Although some oxidation occurred at this concentration it had almost completely ceased. As shown earlier the concentrations of ammonium salts limiting growth are much higher. Rapid oxidation occurred at 2.5 per cent ammonium chloride although it was somewhat depressed. Some oxidation took place at 5. per cent ammonium sulfate. The sulfur organism is much more tolerant to ammonium salts than are nitrifying bacteria. There would seem to be very little ionization of ammonium sulfate in the acid sulfur medium and ionized ammonia is what apparently exerts the particular injury to nitrifying bacteria according to Meyerhoff (1916 a and b, 1917). Even 0.005 N ammonium sulfate injured respiration of nitrate formers 30 per cent at pH 8 to 8.5. Injury was not as great to nitrite formers; a concentration of 0.01 molar ammonium sulfate was slightly injurious to respiration and injury was 90 per cent at 0.1 molar.

From the results of these experiments it is concluded that nitrate is not utilized by the bacterium while the ammonium ion is a very available source of nitrogen.

The inhibitory effects of nitrate on oxidation of sufur in composts may be interpreted in the light of the action of nitrates on *Thiobacillus thiooxidans*—the agent active in this oxidation. In no instance in the course of the many experiments conducted

TABLE 7

Carbon assimilation in the absence of ammonium nitrogen and in the presence of sodium nitrate

DAYS OF INCUBATION	PER CENT $NaNO_3$	CONTROL*	pH	TITER	NITRATE NITROGEN IN 100 cc.		TOTAL C, AS C IN 100 cc.				TOTAL SULFATE, AS S IN 100 cc.				S/C	
					Per culture	Decrease from control	1	2	Average	After subtracting control	1	2	Average	After subtracting control	Per culture	Average
					mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*		
0		+	4.7	1.50	0		3.045	2.584			8.240	7.555				
4		+	4.7	1.50	0		2.599	2.584			6.130	6.525				
19		+	4.6	1.50	0		2.092	2.338	2.540		8.925	8.240	7.603			
14	0	−	2.0	2.95	0	0	2.784	3.015	2.900	0.360	57.340	57.685	57.513	49.910	138.64	
21		−	1.6	4.05	0	0	3.107	3.045	3.076	0.536	89.270	87.898	88.584	80.981	151.09	
26		−	1.4	6.55	0	0	3.491		3.491	0.951	170.305	172.020	171.163	163.560	171.99	
30		−	1.6	4.70	0	0	3.345	3.269	3.307	0.767	112.275	110.330	111.303	103.700	135.20	149.23
0		+	4.7	1.50	6.42		2.769	2.323			7.895	7.555				
7		+	4.7	1.50	6.42		2.215	2.246			6.525	6.525				
26		+	4.7	1.55	7.24		2.123	2.061	2.289		8.925	8.925	7.725			
10	0.05	−	2.0	2.80	6.42	0.27	2.307	2.953	2.630	0.341	49.787	50.816	50.302	42.577	124.86	
14		−	1.8	3.45	6.42	0.27	3.061	3.045	3.053	0.764	74.163		74.163	66.438	86.96	
19		−	1.8	3.65	6.42	0.27	2.845	2.938	2.892	0.603	79.314	78.283	78.799	71.074	117.87	
30		−	1.8	3.75	7.02	−0.33	2.887	3.239	3.063	0.774	86.868	86.180	86.524	78.799	101.81	107.88
0		+	4.7	1.50	36.04		2.615	2.446			7.210	8.240				
15		+	4.7	1.50	34.38		2.215	2.138			7.210	8.240				
27		+	4.7	1.50	38.81		2.184	2.492	2.348		8.584	8.584	8.011			
12	0.25	−	2.0	3.00	24.95	11.46?	2.630	2.830	2.730	0.382	59.057	58.370	58.714	50.703	132.73	
15		−	2.0	2.90	34.38	2.03	2.569	2.999	2.784	0.436	55.624	54.936	55.280	47.269	108.42	
24		−	2.4	2.80	36.59	−0.18	2.584	2.615	2.600	0.252	50.129	49.787	49.958	41.947	166.46	135.87

* + indicates uninoculated controls; − indicates inoculated cultures.

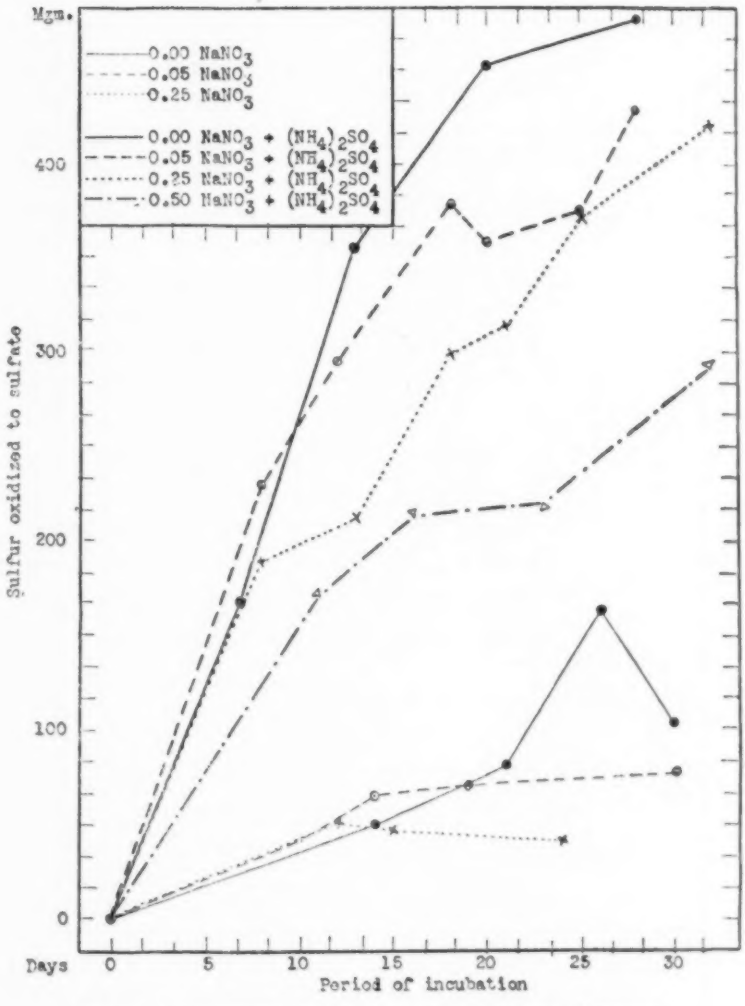


FIG. 2. AVAILABILITY OF NITRATE AND AMMONIUM NITROGEN

TABLE 8

Carbon assimilation in the presence of ammonium nitrogen (0.04 per cent $(NH_4)_2 SO_4$) and sodium nitrate

DAYS OF INCUBATION	PER CENT $NaNO_3$	CONTROL*	pH	TITER	NITRATE NITROGEN IN 100 CC.		TOTAL C, AS C IN 100 CC.				TOTAL SULFATE, AS S IN 100 CC.				S/C	
					Per culture	Decrease from control	1	2	Average	After subtracting control	1	2	Average	After subtracting control	Per culture	Average
					mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.		
0	0	+	4.7	1.50	0		2.292	2.083			16.82	16.48				
5		+	4.7	1.50	0		2.384	2.446	2.301		16.82	16.82	16.74			
7		−	−1.2	6.75	0	0	7.491	8.244	7.868	5.567	183.70	183.70	183.70	166.96	29.99	
13		−	−1.2	12.65	0	0	14.458	14.089	14.274	11.973	372.54	372.54	372.54	355.80	29.72	
20		−	−1.2	15.65	0	0	16.350	16.335	16.343	14.042	469.02	468.69	468.86	452.12	32.20	
28		−	−1.2	16.45	0	0	16.734	16.327	16.531	14.230	495.46	496.49	495.98	479.24	33.68	31.40
1	0.05	+	4.7	1.50	5.99		2.307	2.153			16.82	14.43				
16		+	4.7	1.50	6.42		2.076	1.984	2.130		17.17	16.14	16.29			
8		−	−1.2	8.80	5.89	0.32	9.275	10.044	9.660	7.530	244.81	245.80	245.31	229.01	30.41	
12		−	−1.2	10.70	4.99	1.22	11.520	11.197	11.359	9.229	311.08		311.08	294.79	31.94	
18		−	−1.2	13.35	4.99	1.22	11.628	13.166	12.397	10.267	393.83	393.83	393.83	377.54	36.77	
20		−	−1.2	12.70	6.07	0.14	13.258	13.043	13.151	11.021	375.63	373.57	374.60	358.31	32.50	
25		−	−1.2	13.10	5.82	0.39	13.135	13.305	13.220	11.090	388.68		388.68	372.39	33.58	
28		−	−1.2	14.80	5.99	0.22	14.858	15.089	14.974	12.844	440.52	446.70	443.61	427.32	33.27	33.08
2	0.25	+	4.7	1.50	28.69		2.153	2.215			16.48	17.17				
16		+	4.7	1.50	35.64		2.369	1.984	2.180		16.48	16.48	16.65			
8		−	−1.2	7.35	36.93	−4.76	7.875	7.752	7.814	5.634	204.96	206.01	205.49	188.84	33.52	
13		−	−1.2	8.05	30.43	1.74	8.629	8.444	8.537	6.357	228.67		228.67	212.02	33.35	
18		−	−1.2	10.80	32.37	−0.20	10.659	10.751	10.705	8.525	316.23	316.23	316.23	299.58	35.14	

21		−	−1.2	11.30	29.94	2.23	10.044	9.782	9.913	7.733	332.37	330.66	331.47	314.82	40.71	
25		−	−1.2	13.00	33.27	−1.10	11.659	11.536	11.598	9.418	389.71	291.42	390.57	373.92	39.70	
32		−	−1.2	14.40	33.27	−1.10	12.939	13.198	13.069	10.889	438.80	435.03	436.92	420.27	38.60	36.84
5		+	4.6	1.20			2.336	3.068	2.702		18.88		18.88			
11		−	−1.2	6.80			6.100	6.065	6.083	3.382	188.82	191.02	189.92	171.04	50.60	
16	0.50	−	−1.2	8.30			6.862	7.222	7.042	4.340	231.39	232.97	232.18	213.30	49.15	
23		−	−1.2	7.90			7.423	8.262	7.843	5.141	240.52	237.71	239.12	220.24	42.85	
32		−	−1.2	10.10			8.464	8.498	8.481	5.779	307.96	312.28	310.12	291.24	50.40	48.25

* + indicates uninoculated controls; − indicates inoculated cultures.

with this organism have I observed any increase in oxidation due to either sodium, potassium, or calcium nitrates added to the medium. There will be no attempt made to explain the discrepancy between the results presented here and those reported by the authors mentioned above.

The investigation of growth of the bacterium in the presence of nitrate has also been extended to studies of carbon assimilation. The organism was grown in a medium receiving no addition of nitrogen, in media containing 0.05 and 0.25 per cent sodium nitrate, in a medium containing only ammonium nitrogen, and in media containing ammonium nitrogen in addition to 0.05, 0.25, and 0.50 per cent sodium nitrate. The cultures were analyzed for nitrate as well as for sulfate, carbon, and acidity. The data are presented in tables 7 and 8 and figure 2.

In the absence of any ammonium nitrogen, except what might have been introduced as impurity, there was some oxidation of sulfur and slight amounts of carbon were assimilated. From about 0.25 to 1 mgm. of carbon was assimilated per 100 cc. of medium by the oxidation of 87 to 100 mgm. of sulfur within thirty days. The results indicate that the economy of the utilization of energy by the bacterium for assimilation of carbon is very low under the unfavorable condition of nitrogen starvation. Where no nitrogen was added, the sulfur-carbon ratio was 149.23. In the presence of 0.05 and 0.25 per cent sodium nitrate the ratios were 107.88 and 135.87 respectively. More sulfur was oxidized and more carbon was assimilated in the absence of nitrate than in its presence and the larger amounts of nitrate exerted a greater depressing effect on oxidation and growth. These results are in harmony with those presented in table 6. The injury from sodium nitrate is apparently quite similar to that from potassium nitrate. A glance at the results of the nitrate determinations clearly reveals the fact that none of the nitrate has been consumed by the organism during growth. The slight variations are within the error of the method of analysis except in one instance where some unknown factor affected the results.

In the presence of 0.04 per cent ammonium sulfate, the metabolism of the organism seems changed. Large amounts of sul-

fur were oxidized and carbon assimilated. From 167 to 480 mgm. of sulfur were oxidized in the assimilation of 3.4 to 14.2 mgm. of carbon within the space of a month. Carbon assimilation and oxidation are much more rapid in cultures of this organism than in cultures of nitrifying bacteria. Coleman (1908) reported from 5 to 8 mgm. of carbon in 125 cc. of culture after incubation for three months. Meyerhoff found about 3 mgm. of carbon in 100 cc.

The sulfur-carbon ratios differed from the normal in proportion to the extent of the injury which the nitrate exerted on growth. Metabolism proceeded according to the normal ratio of oxidation to growth in the normal medium. The factor determined (31.40) is almost identical with the factors previously determined (31.82 and 31.73). The ratios in the presence of 0.05, 0.25, and 0.50 per cent sodium nitrate are higher and the extent of the increase in the ratios bear simple proportions to the amounts of nitrate present and the injury produced. The amounts of sulfur oxidized and carbon assimilated are lower in the presence of nitrate than in its absence and the depression effected is greater in the presence of larger amounts of nitrate. This is expressed graphically in figure 2. In the presence of 0.05, 0.25, and 0.50 per cent sodium nitrate the sulfur-carbon ratios are 33.08, 36.84, and 48.25 respectively. The decreases in nitrate in these cultures as well as in those from the preceding experiment are not significant and show no correlation with growth of the organism.

The injurious effects of the nitrate anion are specific and not explained by any osmotic effects they may have created. This is apparent from the low concentrations of nitrate which inhibit growth as compared with the high concentrations of other substances (H_2SO_4 and $MgSO_4$) which are tolerated.

Böttger (1921) has made a somewhat extensive study of the toxic effects of the nitrate radical on many different organisms. Some of the points brought out in his review should be mentioned here. Nitrate prevented nodule formation in roots of legumes. This may not be a direct toxic effect upon nodule bacteria or upon the plant as has been indicated more recently. In considerable

amounts, nitrate was injurious to organisms decomposing cellulose. At high concentrations of nitrate, non-spore-forming bacteria are replaced by spore formers in the soil. It has been noted that nitrates exerted an injurious effect on sewage purification. The author compared the effects of concentrations of nitrates on growth of yeasts, fungi, and bacteria with the effects produced by magnesium sulfate in concentrations which would produce the same osmotic effects in the solutions as the nitrates. Many of the organisms studied were remarkably tolerant to high concentrations of nitrates. The results of the investigations led to the conclusions that the nitrate anion exerted an injury on yeasts, fungi, and bacteria which was specific and not due to the cations introduced with the nitrate or to osmotic effects produced by the concentrations used. In cases where organisms used nitrate as a source of nitrogen, some stimulation was noted in a few instances at low concentrations. For alcohol-forming yeasts which do not attack the nitrate, no stimulating effects were apparent from small amounts of nitrate. Injury to vegetation as well as fructification of fungi was proportional to the concentration of the nitrate. In general, nitrates of the alkaline earths (Ca) were more toxic to all of the organisms than nitrates of the alkalies (K, Na).

Some organisms are apparently indifferent to considerable concentrations of nitrate. Denitrifying bacteria were not apparently sensitive to the anion. Meyerhoff (1916 a and b) found that respiration of nitrate-formers studied for short intervals, continued at concentrations of 10 to 20 per cent sodium nitrate! Meyerhoff concluded as a result of his investigations that, ". . . . Ja, NO_3 muss geradezu als das unshädlichste Anion bezeichnet werden, das sich überhaupt den Nitratbakterien gegenüber finden lässt" (p. 404). Nitrate was somewhat more injurious to the nitrite formers.

This specific injurious effect of the nitrate anion which has been observed by Böttger for so many organisms has strikingly manifested itself in its effects on both oxidation and growth of *Thiobacillus thiooxidans*.

Availability of urea

The disappearance of glucose in the medium suggested its possible consumption by the organism. Providing such was the case, some simple organic compounds of nitrogen might also be attacked. Urea was introduced into the medium as such a source of nitrogen, since it is a relatively simple organic compound with nitrogen bound up in a form similar to the nitrogen in inorganic ammonium compounds. One concentration of urea (0.02 per cent) was used. In one medium it was introduced as the

TABLE 9

Availability of urea as a source of nitrogen

TREATMENT	TITER, AFTER*					
	5 days		10 days		18 days	
	Per culture	Average	Per culture	Average	Per culture	Average
None	0.50		1.40		2.05	
	0.85	0.68	1.50	1.45	2.50	2.28
0.02 per cent urea	1.20		2.20		3.25	
	1.15	1.18	2.15	2.18	3.05	3.15
0.04 per cent $(NH_4)_2 SO_4$	0.00		7.10		12.25	
	5.00	2.50	10.30	8.70	12.10	12.18
0.02 per cent urea + 0.04 per cent $(NH_4)_2SO_4$	3.95		11.00		12.90	
	0.10	2.03	5.20	8.10	9.30	11.10

*Increases over uninoculated media.

only source of nitrogen and in another, ammonium sulfate was also included. As controls, media were used which received no addition of nitrogen and which contained only ammonium nitrogen. In the preparation of these media, a standard solution of urea was sterilized by passage through a Berkefeld filter since urea is decomposed by heat. Portions of the sterile filtrate were added to the sterilized sulfur media before inoculation. The results are presented in table 9.

There was feeble oxidation in the medium receiving no addition

of nitrogen and in the medium containing urea alone, slightly more oxidation in the urea medium. This increase is accounted for by the slight amount of ammonia introduced as impurity with the urea. As determined by Nesslerization, both the urea medium and that receiving no addition of nitrogen contained

TABLE 10

*Oxidation of sulfur in the presence of pepton**

PER CENT OF PEPTON ADDED	pH, AFTER†			TITER, AFTER			S AS SULFATE IN 100 CC., AFTER		
	4 days	8 days	14 days	4 days	8 days	14 days	4 days	8 days	14 days
Lacking $(NH_4)_2SO_4$									
							mgm.	*mgm.*	*mgm.*
0	2.0	1.6	1.5	1.20	1.95	2.90	37.09	64.56	93.40
0	2.0	1.8	1.6	1.10	1.70	2.50	33.52	56.32	75.00
0.01	1.9	1.5	1.4	1.30	2.55	4.05	41.21	72.41	121.69
0.01	1.6	1.4	1.3	2.00	3.45	5.25	64.56	104.94	157.96
0.05	1.7	1.3	1.4	1.70	2.90	4.00	53.57	91.48	123.07
0.05	1.6	1.4	1.4	2.20	3.60	4.65	68.13	109.88	139.28
0.25	1.7	1.4	1.4	2.10	3.25	4.40	65.93	103.01	134.87
0.25	2.2	1.9	1.6	1.10	2.05	2.95	34.34	67.30	87.90
1.25	3.8	3.0	2.6	0.60	1.00	1.65	16.21	29.39	45.32
1.25	4.7	3.6	3.4	0.25	0.00	1.10	7.42	18.40	31.32
$(NH_4)_2SO_4$ present									
0	1.5	−1.2	−1.2	3.55	7.00	8.70	109.05	227.17	264.26
0	1.5	−1.2	−1.2	3.75	7.80	9.45	113.17	244.21	291.63
0.01	1.3	−1.2	−1.2	4.45	6.95	8.35	137.35	218.39	252.72
0.01	1.2	−1.2	−1.2	4.85	8.90	10.65	145.59	276.63	320.03
0.05	1.0	1.2	1.2	2.55	4.15	5.20	79.66	124.16	160.16
0.05	1.6	1.3	1.2	2.75	4.35	5.25	85.16	131.03	167.57
0.25	2.0	1.9	1.7	1.55	2.05	2.85	45.33	68.13	90.10
0.25	1.8	1.6	1.5	2.15	3.05	3.90	68.68	65.93	124.99
1.25	3.9	3.2	3.0	0.50	0.80	1.25	17.86	31.04	47.80
1.25	3.7	3.0	2.6	0.70	1.05	1.70	48.90	19.78	51.37

* Data are given as increases over control.

† Original pH of media: 0 pepton 4.7, 0.01 pepton 4.6, 0.05 pepton 4.8, 0.25 pepton 5.2, 1.25 pepton 5.6.

small amounts of ammonium nitrogen, the urea medium giving a slightly higher amount. Oxidation was initially delayed in one of the duplicate cultures of both the normal medium and the medium containing both urea and ammonium sulfate. Oxida-

tion was the same in both of these media and urea exerted no apparent depressing or stimulating effect at the concentration of 0.02 per cent. Urea is not apparently attacked by *Thiobacillus thiooxidans* and consequently is not available to the organism as a source of nitrogen.

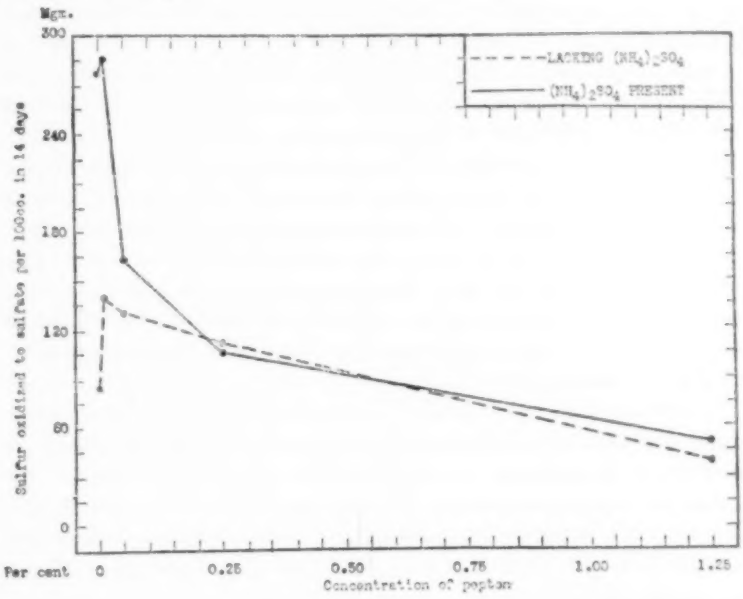


FIG. 3. INFLUENCE OF PEPTON ON OXIDATION IN THE PRESENCE AND ABSENCE OF $(NH_4)_2SO_4$

Oxidation of sulfur in the presence of pepton

Media were used containing Difco pepton in concentrations of 0.01, 0.05, 0.25, and 1.25 per cent both in the presence and absence of ammonium nitrogen. The results of the experiment are recorded in table 10 and figure 3.

In the absence of ammonium nitrogen, oxidation was retarded as compared with oxidation in the presence of ammonium sulfate. Considerable growth occurred however, in both media. The Difco pepton is composed of many different compounds and no

doubt contained some ammonium nitrogen or some ammonium nitrogen was split off during sterilization. Such ammonium nitrogen may have been responsible for the increased oxidation in the presence of small amounts of pepton and in the absence of ammonium sulfate. No conclusions can be drawn from this experiment concerning the consumption of any of the compounds which compose pepton. The effects of some concentrations of pepton on oxidation are, however, indicated by the results. It has been shown previously (Waksman and Starkey, 1923) that oxidation is inhibited in a concentration of 2.5 per cent pepton. From the data presented it is apparent that, in the absence of ammonium sulfate, oxidation is enhanced by small amounts of pepton. No injurious effects are apparent at 0.05 per cent. Even at 0.25 per cent, injury is not great. At 1.25 per cent, depression is pronounced. The same effects are evident in the presence of ammonium sulfate with the exception that as vigorous oxidation occurs in the absence of pepton as in its presence even in low concentrations.

Providing that the organism has the capacity to attack pepton, there should be an increase in the amino acid content of the media as growth progresses. Determinations for amino acids in cultures at different periods of incubation failed to reveal any increase in the amino acid content. In the presence of pepton, the assimilation of carbon is higher than in the normal medium, averaging 53.13.

Glycocoll completely inhibits oxidation at the concentration of 1 per cent and considerably depresses it at 0.5 per cent. Should the organism consume glycocoll it would be expected that there would be a decrease in the amino acid content of cultures containing this amino acid. No decrease in the amino acid content of cultures containing glycocoll has been noted from numerous determinations.

Although the data available are not sufficient to definitely prove the point, all results indicate that amino acids are not available either as sources of nitrogen or carbon. Winogradsky (1904–6) states that nitrite-formers are unable to attack proteins and amino compounds and can effect no oxidation of nitrogen-

ous substances. Meyerhoff (1916 a and b, 1917) found the amino acids as injurious as ammonium compounds to nitrifying bacteria. These compounds were more toxic at more alkaline reactions.

Conclusions drawn by others that organic nitrogen is available to the organism seem to be without adequate foundation.

DISCUSSION

Thiobacillus thiooxidans exhibits many physiological characteristics which are common to autotrophic bacteria, but exhibits others which distinguish it from any other organism yet known.

The sources from which energy is derived are not different for this sulfur bacterium from those utilized by many other sulfur bacteria. The effects of concentrations of some inorganic compounds are not strikingly different on growth of any of the organisms. The sources of carbon and oxygen are the same for most of the autotrophic bacteria known.

Thiobacillus thiooxidans is outstanding in its indifference to high concentrations of acid. None of the other autotrophic bacteria develop well in acid media and their best growth is obtained in the alkaline range. Growth of this particular sulfur organism is inhibited in alkaline solution media and is most rapid at the very acid range of pH 2.0 to 3.0. In the late stages of oxidation, after large amounts of acid have accumulated, two factors are operative which tend to retard development. The influences of either a highly concentrated solution or an abundance of hydrogen-ions must be considerable. Both of these agents together should exert an appreciably greater effect than the sum of the effects which might be produced by either one alone. The organism is surprisingly tolerant to the combined effects of both together.

It is somewhat more economical in the utilization of energy than the nitrifying bacteria.

The nitrate-former exhibits marked tolerance to high concentrations of the end product of its oxidation process (nitrate). Unlike this organism, the sulfur bacterium is very sensitive to the nitrate anion. However, the end product of sulfur oxidation (sulfate) is tolerated by *Thiobacillus thiooxidans* in as high con-

centration as 20 per cent in the form of magnesium sulfate. The tolerance of both organisms to the end products of their oxidation processes is noteworthy.

Glucose supports no growth of the organism as the sole source of energy or carbon. The disappearance of this carbohydrate in the presence of inorganic sulfur as the source of energy is, however, consistent. The organism resembles nitrifying bacteria in this as in many other respects. Whether the organisms would transform or consume other organic compounds as they do glucose is merely speculative.

Coincident with depression of oxidation caused by various factors, such as concentrations of acid, nitrate, or pepton, there is a decrease in the efficiency of the utilization of the energy available for assimilation of carbon. This indicates that there has been a detrimental modification in the metabolism of the organism. The extent of the decrease in this efficiency is closely associated with the extent of the injury to oxidation which was exerted by these different factors.

SUMMARY

1. The results of experiments concerned with glucose in sulfur media used for the cultivation of an autotrophic sulfur-oxidizing bacterium, *Thiobacillus thiooxidans*, may be summarized as follows:

a. Glucose disappears from the sulfur medium in the presence of the developing organism during incubation and there is a general correlation between the amount of glucose which disappears and the amount of sulfuric acid produced, which is, in turn, proportional to growth. There is, however, no consistent correlation between the initial concentration of the glucose and the amounts which disappear during growth of the organism.

b. The acid produced by the oxidation of sulfur is not alone responsible for the disappearance of glucose in the cultures.

c. No growth takes place in the presence of glucose in the absence of sulfur or sulfur compounds available as sources of energy.

d. The bacterial cells and cell products do not interfere with the glucose in a physical way.

e. Berkefeld filtrates from cultures of the organism fail to effect significant transformation of the glucose. It seems apparent from these experiments that glucose, in small amounts, may enter into the metabolism of the cells in the presence of sulfur as a source of energy and may be used either as a source of energy or carbon for growth, or both.

2. Citric acid inhibits growth at a concentration of 5 per cent but oxidation is active in the presence of 2.5 per cent.

3. The experiments concerned with the utilization of nitrate and ammonium nitrogen may be summarized as follows:

a. There is some oxidation in media receiving no nitrogen other than what may have been absorbed from the air or introduced as impurity in the non-nitrogenous compounds incorporated in the medium.

b. Ammonium nitrogen is the only source of nitrogen that has been found to be available to the organism. More rapid oxidation was never observed in the presence than in the absence of nitrates and oxidation was depressed in media containing nitrate in even as low concentration as 0.05 per cent KNO_3. About 1 per cent KNO_3 completely inhibited oxidation.

c. The amount of sulfur oxidized per unit of carbon assimilated in a medium to which no nitrogen was added except as impurity is 149. In the presence of 0.05, and 0.25 per cent sodium nitrate as the only source of nitrogen, the ratios were 108 and 136 respectively. In the presence of ammonium sulfate in addition to 0.05, 0.25, and 0.50 per cent sodium nitrate, the ratios were 33, 37, and 48 respectively. The decrease in the economy of utilization of energy is proportional to the amount of nitrate added. The injurious effects of nitrate are specific for the anion and not due to any great extent to the osmotic effects which might be created by the concentrations of the nitrate.

4. Urea is not attacked and does not appear to be available to the organism as a source of nitrogen.

5. Oxidation is inhibited in the presence of 2.5 per cent of pepton and injury is pronounced at 1.25 per cent. Results indicate that neither pepton nor amino acids are available either as sources of nitrogen or carbon.

ACKNOWLEDGMENTS

The author is particularly desirous at this time of expressing his sincere appreciation to Dr. J. G. Lipman, Dr. S. A. Waksman and the associates who have aided through suggestions, continued interest, or material assistance in the performance of this work and that reported in a preceding contribution (1925). The work has been performed under the close surveillance of Dr. Waksman whose ready and abundant suggestions were at all times available. The stimulation and encouragement created by his interest, knowledge of the field, and guidance in the work have been of invaluable advantage to the author.

REFERENCES

ALLISON, R. V. 1923 A preliminary note on the efiect of the nitrate radical upon biological oxidation of inorganic sulfur. N. J. Agr. Exp. Sta. Rept. for 1922, p. 366–369.

BERTRAND 1906 Bull. Soc. Chim. de France, **35**, 1285. Cf. Hawk, Physiological Chemistry, sixth ed., 1920, p. 550–553, Blakiston, Phila.

BÖTTGER, H. 1921 Ueber die Giftwirkungen der Nitrate auf niedere Organismen. Centralbl. Bakt. (etc.), Abt. 2, **54**, 220–261.

COLEMAN, L. C. 1908 Untersuchungen über Nitrifikation. Centralbl. Bakt. (etc.), Abt. 2, **20**, 401–420, 484–513.

JOFFE, J. S. 1922 Biochemical oxidation of sulfur and its significance to agriculture. N. J. Agr. Exp. Sta. Bul. 374, 91 p.

LIPMAN, J. G., WAKSMAN, S. A., AND JOFFE, J. S. 1921 The oxidation of sulfur by soil microörganisms. I. Soil Sci., **12**, 475–489.

MCLEAN, H. C. 1918 The oxidation of sulfur by microörganisms in its relation to the availability of phosphates. Soil Sci., **5**, 251–290.

MEYERHOFF, O. 1916a Untersuchungen über den Atmungsvorgang nitrifizierender Bakterien. I. Die Atmung des Nitratbildners. Archiv. f. d. ges. Physiol., **164**, 353–427.

MEYERHOFF, O. 1916b Untersuchungen über den Atmungsvorgang nitrifizierender Bakterien. II. Beeinflussigen der Atmung des Nitratbildners durch chemische Substanzen. Archiv. f. d. ges. Physiol., **165**, 229–284.

MEYERHOFF, O. 1917 Untersuchungen über den Atmungsvorgang nitrifizierender Bakterien. III. Die Atmung des Nitritbildners und ihre Beeinflussung durch chemische Substanzen. Archiv. f. d. ges. Physiol., **166**, 240–280.

STARKEY, R. L. 1925 Concerning the physiology of *Thiobacillus thiooxidans*, an autographic bacterium oxidizing sulfur under acid conditions (preceding paper).

WAKSMAN, S. A., AND JOFFE, J. S. 1922a The chemistry of the oxidation of sulfur by microörganisms to sulfuric acid and transformation of insoluble phosphates into soluble forms. Jour. Biol. Chem., **50**, 35–45.

WAKSMAN, S. A., AND JOFFE, J. S. 1922b Microörganisms concerned in the oxidation of sulfur in the soil. II. *Thiobacillus thiooxidans*, a new sulfur-oxidizing organism isolated from the soil. Jour. Bact., **7**, 239–256.

WAKSMAN, S. A., AND STARKEY, R. L. 1922 Carbon assimilation and respiration of autotrophic bacteria. Proc. Soc. Exper. Biol. and Med., **20**, 9–14.

WAKSMAN, S. A., AND STARKEY, R. L. 1923 On the growth and respiration of sulfur-oxidizing bacteria. Jour. Gen. Physiol., **5**, 285–310.

WINOGRADSKY, S. 1904–6 Nitrification. Handbuch der Technischen Mykologie (Lafar) 3. Mykologie des Bodens, des Wassers, und des Düngers. Part 1, chap. 2, p. 132–192, Fischer, Jena.

WINOGRADSKY, S., AND OMELIANSKY, W. 1899 Ueber den Einfluss der organischen Substanz auf die Arbeit der nitrifizierenden Mikrobien. Centralbl. Bakt. (etc.), Abt. 2, **5**, 329–343, 377–387, 429–440.

CONTRIBUTIONS TO THE KNOWLEDGE OF VIBRIO CHOLERAE

I. FERMENTATION OF CARBOHYDRATES AND POLYATOMIC ALCOHOLS BY VIBRIO CHOLERAE

KEIZO NOBECHI

From the third Section of the Bacterio-Serological Department of the Institute for Infectious Diseases of Tokio Imperial University; Director of the Institute Prof. M. Nagayo

Received for publication September 19, 1924

INTRODUCTION

The ability to ferment sugars, as well as the exhibition of specific immune reactions are of primary importance in the classification of bacteria, especially in the case of the streptococci, the *Micrococcus catarrhalis* group, and the colon-typhoid-dysentery group.

The cholera vibrio is distinguished from non-choleraic vibrios by its immune reactions, but no cultural method capable of differentiating them has yet been found. Hemolysin production was previously considered to be specific to non-choleraic spirilla, but now it is known to be a general potential power common to all spirilla, including *V. cholerae*, differing in intensity with each strain. Most of the spirilla commonly display a positive cholera red reaction. Production of a proteolytic enzyme is also one of their common features, and they have also a generally higher alkali tolerance than most other bacteria.

If we could find a method to differentiate *V. cholerae* from non-choleraic spirilla, culturally, it would form a valuable contribution of practical significance. Various kinds of non-choleraic spirilla live in dirty sea and river water, sometimes abundantly. The author came to realize the lack of a selective medium for differentiating the *V. cholerae* from other non-choleraic spirilla,

in his study as to the fate of *V. cholerae* in sea and river water. As is well known, the spreading of cholera epidemics is directly connected with the water, and studies on the behavior of *V. cholerae* in water are therefore of great importance.

There have been frequent discussions in regard to the alteration of the specific immune character of *V. cholerae* in sea and river water. Opinions are widely divergent regarding the term of life of *V. cholerae* in natural sea and river water. As an aid in the solution of those important problems, selective media to differentiate *V. cholerae* from non-choleraic spirilla would be of great service.

In the colon-typhoid dysentery group many studies have been made concerning the behavior of these organisms towards various carbohydrates and polyatomic alcohols. The writer hopes to determine first of all whether *V. cholerae* and non-choleraic spirilla can be distinguished in this way; and secondly whether varieties exist in this field among *V. cholerae* strains, as is true in the case of streptococci, or dysentery bacilli, and above all, whether the immunological varieties, the original and the varied types of Kabeshima (1913; 1918a; 1918b), and the middle type of the author, of which descriptions will be given later, also differ in sugar-splitting activities.

We could find no treatise specifically dealing with *V. cholerae* or with spirilla in general, in relation to the action of sugar fermentations except some notes of experiments on a small scale employing a limited number of the common sugars with only a few strains.

The amylolytic activity of *V. cholerae* was, however, studied long ago by Bitter (1896), Fermi (1890) and Gordon (1905). Kodama (1921), applying this character, prepared selective media for *V. cholerae*—amylum litmus agar, amylum fuchsin agar and amylum fuchsin cattle serum agar. The bean gruel litmus medium was previously invented by Yamada (1919) as a selective medium for *V. cholerae;* he gives, however, no consideration to the chemical processes displayed on this medium. The fermentation of bean starch by cholera germs may perhaps determine the specific character of this medium. Oyama (1921) investigated

the relations of the amylum fermentation and the nitrite formation of *V. cholerae*.

Studies upon the invertase formation by *V. cholerae* were made long ago by Sclavo (1892), Fermi and Montesano (1896) and Kuprianow (1893). Selective media for *V. cholerae* containing sucrose, devised by Bendick (1912) and Aronson (1915), are based upon this character of the cholera organism. In another medium containing sucrose prepared by Teague and Travis (1916) for the selection of *V. cholerae*, the sucrose serves only as a growth-promoting factor, and the splitting of this sugar is not the main source of color change.

Aronson (1915) states that cholera germs ferment nearly all carbohydrates which he has studied: sucrose, glucose, maltose, mannitol, lactose, amylum and dextrin, and he notes especially that while the fermentation of sucrose is very intensive with *V. cholerae*, most of the common intestinal bacteria do not decompose this sugar. This is the reason that he recommended sucrose media for the elective cultivation of *V. cholerae*. Violle (1919) notes in his monograph, that *V. cholerae* ferments all carbohydrates and polyatomic alcohols: glycogen, dextrin, sucrose, maltose, lactose, glucose, levulose, galactose, mannitol, erythritol and dulcitol with production of acid, but fails to form gas, while amidon is not fermented by this germ.

No investigations concerning fermentation of other sugars and alcohols by *V. cholerae* have been found.

The author at first studied the fermentation of twelve carbohydrates, four polyatomic alcohols and one glycoside by *V. cholerae* and non-choleraic spirilla, using twenty-one strains of *V. cholerae*, and eight strains of non-choleraic spirilla differing in age and source.

The positive fermentation of sucrose was proved to be one of the common characters of *V. cholerae*. One strain "Koch" lacks, however, this ability—forming a remarkable exception, for its colonies on Aronson's medium, which is increasingly employed, are colorless, while other strains invariably form red colonies upon this selective medium. The author performed numerous experiments and observations on "Koch" to determine whether this

anomaly of "Koch" is temporary or permanent. With other bacteria, especially of the colon-typhoid dysentery group, variations and reversions of sugar fermentation, occurring spontaneously or induced artificially, are not infrequently reported.

EXPERIMENT I. FERMENTATION OF CARBOHYDRATES AND POLYATOMIC ALCOHOLS BY V. CHOLERAE AND OTHER SPIRILLA

1. Source and age of the strains of V. cholerae and other spirilla used in the experiment

The author used for this examination 21 cholera strains selected from 88 stock cultures, and 8 non-choleraic spirilla, of different ages and various sources as noted in tables 1 and 2.

TABLE 1

Source, age and immunological type of cholera strains

APPELLATIONS	DATES OF ISOLATION	PLACES OF ORIGIN	IMMUNOLOGICAL TYPES
Hamburg	1892	Hamburg	Original
El-Tor	1805	El-Tor, Egypt	Original
Koch		(Koch Institute)	Original
Pasteur		(Pasteur Institute)	Original
Giran	1912	Giran, Formosa	Varied
Yanagiwara	1916	Yokohama	Original
Takahashi	1917	Gumma Prefecture	Original
Boko	1919	Boko, Formosa	Varied
Taniguchi	1920	Kobe	Varied
Inaba	1921	Kanagawa Prefect.	Original
Hikojima I	1921	Hikojima, Moji	Middle
Tsuda	1921	Tsingtao, China	Middle
Ogawa	1921	Nagasaki	Varied
Shanghai I	1922	Shanghai, China	Middle
Fukuoka I	1922	Fukuoka	Middle
Dairan A	1922	Dairen, China	Middle
Kumamoto	1922	Kumamoto	Middle
Oseko	1922	Yamaguchi	Middle
Hanai	1922	Shimane Prefect.	Middle
Katahara	1922	Tokyo	Middle
295	1922	Chiba Prefecture	Middle

TABLE 2

Source and age of non-choleraic spirilla

APPELLATION	DATE OF ISOLATION	PLACE OF ORIGIN
Finkler et Prior	1885	
Deneke	1885	
Metschnikoff	1887	Odessa
Denken		(Our Institute)
Tsingtao	1820	Tsingtao, China
Shimonoseki	1921	Hikejima, Moji
Kobe	1922	Kobe
Kubota	1922	Tokyo

2. Medium

Dunham's pepton water prepared with Witte pepton was divided in flasks; carbohydrates and polyatomic alcohols were added to these flasks to a strength of 1 per cent and then a sufficient quantity of litmus solution, purified almost in the same way as described by Morse (cited by Clark, 1922) as indicator. Each specimen was tubed in small Durham's gas tubes, 2 cc. in each tube, and sterilized discontinuously by fifteen minutes' exposure at 100°C. on each of three consecutive days. Sterility was proved by incubating for two days at 37°C.

The original pH values of litmus free sugar pepton waters prepared and sterilized in exactly the same way, were determined colorimetrically; they read as follows:

Glucose.....7.2
Arabinose.....7.0
Amylum.....7.2
Inulin.....7.4
Mannitol.....7.2
Dulcitol.....7.2
Sugar-free control.....7.2

3. Carbohydrates, polyatomic alcohols and glucoside examined

Twelve carbohydrates and four polyatomic alcohols and a glucoside as tabulated below were used.

Carbohydrates:

Monosaccharides:

Pentoses: Arabinose, xylose
Hexoses: Glucose, levulose, galactose, mannose
Disaccharides: Lactose, sucrose, maltose
Polysaccharides: Dextrin, amylum, inulin

Polyatomic alcohols:

Trihydric: Glycerol
Hexahydric: Mannitol, dulcitol, isodulcitol (rhamnose)
Glycoside: Salicin

Most of these preparations were from Marck (Darmstadt).

4. Observation

Two drops of germ emulsions, made with the strains mentioned above, were implanted in each sugar tube. The emulsions were prepared with heavy inoculations in pepton water to contain germs in quantity of one loopful in each 1 cc. Itakura (1923) recommended the inoculation of the greatest possible numbers of germs to get an accurate result. After inoculation all tubes were put into an incubator, and observations were made during two weeks, keeping the tubes at 37°C.

Two control tubes without inoculation, for each sugar, were incubated together with the others, to see whether any alteration of color tones resulted. Authentic strains of the typhoid bacillus and of paratyphoid A and B bacilli were inoculated in each sugar tube and incubated simultaneously in order to prove the purity of the sugar specimens used. For the inulin a strain ofpneumococcus I type, and for the salicin three strains of *Streptococcus pyogenes* were used as special controls.

Transplantations upon agar plates were made intermittently from the tubes of representative strains, and from those tubes, which showed abnormal reactions, in order to recognize the growth of germs or to find any intercurrent contamination. With the representative strains and with those which reacted abnormally, the same test, using newly prepared media, was again performed, to determine whether the results were constant.

5. Results of experiments

A. Fermentative powers of V. cholerae. i. Acid formation: All strains of *V. cholerae* ferment all hexoses; glucose, levulose, galactose and mannose, but fail to ferment the pentoses; arabinose and xylose. Among disaccharides, maltose and sucrose were split in twenty-four hours by all strains; "Koch," however, did not ferment the sucrose at all, being the single exception. The lactose is fermented in different degrees according to the strains; some did not redden the lactose tubes at all, and some fermented it intensively. Among polysaccharides, dextrin and amylum (soluble) were fermented intensively by all strains, but inulin was not attacked.

Concerning the polyatomic alcohols, trihydric glycerol was, as in the case of lactose, fermented variously with the strains. All strains split mannitol but not dulcitol and isodulcitol among the hexahydric alcohols. Salicin, a glycoside, remained entirely intact.

To summarize the results, glucose, levulose, galactose, mannose, maltose, sucrose, dextrin, amylum and mannitol were decomposed uniformly by all strains, while arabinose, xylose, inulin, dulcitol, isodulcitol and salicin were not at all fermented.

Generally speaking, the tubes containing fermentable sugars assumed the litmus color tone (+++), corresponding nearly to pH = 5.5, after incubation for twenty-four hours. The germs which had been inoculated died out within one or two days, indicating that the culture had attained the physiological constant of hydrogen ion concentration according to Michaelis. Fermentation of galactose and mannitol were slightly delayed and the acidity attained seemed to be a little weaker.

Reversion towards alkalinity from once attained acidity, was never recognized.

The color tones of the tubes containing unaffected sugars turned to bluish from neutral, the alkaline products from the albuminous substances contained in the media perhaps governing the tone; sugar free litmus pepton water also became bluish under the growth of *V. cholerae*. Germs survived well in these tubes until the end of the observation.

TABLE 3A

Fermentation of carbohydrates and polyatomic alkohols by V. cholerae

INGREDIENTS	STRAINS													
	El-Tor		Hamburg		Koch		Pasteur		Giran		Yanagiwara		Takihashi	
Arabinose	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Xylose	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Glucose	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Laevulose	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Galactose	+	+++	+	+++	+++	+++	+	+++	++	+++	++	+++	++	+++
Mannose	−	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Maltose	+++	+++	+++	+++			+++	+++	+++	+++	+++	+++	+++	+++
Lactose	*	++	−	++	−	−	−	++	−	+	−	++	++	++
Sucrose	+++	+++	+++	+++	−	−	+++	+++	+++	+++	+++	+++	+++	+++
Dextrin	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Amylum	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Inulin	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Glycerol	*	++	−	++	−	−	+	++	−	++	*	++	++	+++
Mannitol	+	+++	++	+++	++	+++	++	+++	++	+++	++	+++	++	+++
Dulcitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Isodulcitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Salicin	−	−	−	−	−	−	−	−	−	−	−	−	−	−

The first and the second of the two signs in each plot indicate respectively the reactions after twenty-four hours and two weeks.
Date: March 28, 1922, and April 19, 1923.

TABLE 3B

Fermentation of carbohydrates and polyatomic alkohols by V. cholerae

INGREDIENTS	STRAINS													
	Boke		Taniguchi		Inaba		Hikojima I		Tauda		Ogawa		Shanghai I	
Arabinose	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Xylose	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Glucose	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Laevulose	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Galactose	++	+++	++	+++	++	+++	++	+++	++	+++	++	+++	++	+++
Mannose	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Maltose	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Lactose	+	+	+	+	–	++	+	++	+	++	–	++	–	++
Sucrose	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Dextrin	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Amylum	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Inulin	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Glycerol	+	+++	+	++	+	++	+	++	–	++	+	++	–	++
Mannitol	++	+++	++	+++	++	+++	++	+++	++	+++	++	+++	++	+++
Dulcitol	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Isodulcitol	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Salicin	–	–	–	–	–	–	–	–	–	–	–	–	–	–

TABLE 3C

Fermentation of carbohydrates and polyatomic alkohols by V. cholerae

INGREDIENTS	STRAINS													
	Fukuoka I		Dairen A		Kumamoto		Oseko		Hanai		Katahara		295	
Arabinose	−	−	−−	−−	−−	−	−	−	−	−	−	−	−	−
Xylose	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Glucose	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Laevulose	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Galactose	++	+++	++	+++	++	+++	++	++	++	++	++	+++	++	+++
Mannose	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Maltose	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Lactose	−	++	−	++	−	++	−	++	−	++	−	++	−	++
Sucrose	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Dextrin	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Amylum	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Inulin	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Glycerol	−	++	*	++	*	++	+	++	+	++	*	++	+	++
Mannitol	++	+++	++	+++	+++	+++	++	+++	++	+++	++	+++	++	+++
Dulcitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Isodulcitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Salicin	−	−	−	−	−	−	−	−	−	−	−	−	−	−

The lactose from Merck-Darmstadt and the glycerol from Price-London were fermented variously by different strains, by some entirely negatively (−), but by some weakly, showing a litmus color tone (+) corresponding nearly to pH = 6.5; by some moderately, showing a tone (++) corresponding nearly to pH = 6.0 and by the others distinctly, showing a litmus tone (+++) corresponding nearly to pH $\leqq$5.5.

The examination was repeated using preparations of lactose from Kahlbaum (Berlin) and Gehe (Dresden), and a chemically pure preparation of glycerol of the Nippon Glycerin Kogyo-Osaka; these gave qualitatively equal results, only differing quantitatively from those of the former test.

The historical strains "Hamburg" and "El-Tor," which are over thirty and nearly twenty years old respectively, revealed the same behavior towards all sugars as the newly isolated strains. "Koch's" failure to ferment sucrose cannot, therefore, be due merely to old age.

The sucrose used in the former test was from Merck (Darmstadt). The author also tested the preparations from Kahlbaum (Berlin) and Gehe (Dresden), but they also were not decomposed by "Koch."

No difference in the sugar fermentation was detected among the three immunological varieties (table 3, A, B and C).

ii. Gas production: With all sorts of carbohydrates and polyatomic alcohols, no gas production was ever discernible.

iii. Reduction of the litmus: Müller (1899) has already noticed the intensive reduction of litmus by *V. cholerae*.

In the author's experiment, the tubes, which showed maximal acid production and in which the germs had died out, revealed no detectable reduction of the litmus; while in those tubes, containing arabinose, xylose, inulin, dulcitol, isodulcitol and salicin, which remained negative as to acid formation, the litmus was distinctly reduced. In the latter tubes, germs continued living and propagating so the reduction must be a parallel phenomenon to the life of the cells. The leucoproducts of the reduced litmus could be returned to the colored state by shaking.

B. Fermentation reactions of the non-choleraic spirilla. A re-

markable pnenomena was that all kinds of spirilla behaved almost in the same way as *V. cholerae*. They acidified glucose, levulose, galactose, mannose, maltose, sucrose, dextrin, amylum and mannitol. Most tubes containing the above mentioned ingredients were reddened to the litmus color tone (+++) corresponding to pH $\leqq$ 5.5 in twenty-four hours; germs were soon killed and reduction of litmus was not noticeable in those tubes.

There were, however, exceptions; "Kobe" and "Kubota" failed to ferment the mannose, and this was constant in repeated tests.

A reversion from the once attained intensive acidity towards alkalinity was observed in a few tubes: by "Denken" in the dextrin tube, by "Tsing-tao" and "Kobe" in the mannitol tubes. In those tubes, life of the germs continued, indicating that the physiological constant of the hydrogen ion concentration was not surpassed in those tubes and the alkaline products from the albuminous substances due to the vital activities of the surviving germs began to affect the general reaction.

Arabinose, xylose, inulin, dulcitol, isodulcitol and salicin were here also not decomposed, and the litmus tone turned gradually towards alkalinity. In these cases reduction of the litmus was distinctly noticed and the germs continued their propagation; "Shimonoseki" formed an exception showing positive fermentation of arabinose, and this also proved constant in repeated test.

The lactose and glycerol were split variously by different strains, as in the case of *V. cholerae*.

Gas formation was not detected with the non-choleraic spirilla (table 4).

C. Control test. In examining the mode of fermentation by typical strains of the typhoid bacillus and of paratyphoid A and B bacilli used as control, it appears that the chemical nature of the sugars corresponds entirely to that of the carbohydrates and polyatomic alcohols used hitherto by other authors. Further, the inulin was decomposed by a strain of pneumococcus I type, and the salicin tube was acidified by *Streptococcus pyogenes* strains, though these ingredients remained unaffected by *V. cholerae* and other spirilla (table 5).

TABLE 4

Fermentation of carbohydrates and polyatomic alkahols by non-choleraic spirilla

SUGAR	STRAIN															
	Finkler Prior		Deneke		Metschnikoff		Denken		Tsingtao		Shimonoseki		Kobe		Kubota	
Arabinose	−	−	−	−	−	−	−	−	−	−	+++	+++	−	−	−	−
Xylose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Glucose	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++++	+++	+++	+++
Laevulose	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Galactose	++	++	++	++	++	+++	++	++	++	+++	+++	+++	++	+++	++	+++
Mannose	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	−	−	−	−
Maltose	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Lactose	−	++	−	++	−	+	−	+	−	++	−	*	−	−	−	+
Sucrose	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Dextrin	+++	+++	+++	+++	+++	+++	+++	−	+++	+++	+++	+++	+++	+++	+++	+++
Amylum	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	−	+++	+++
Inulin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Glycerol	+	++	*	++	+	++	*	++	+	+	*	*	−	+	−	−
Mannitol	+++	+++	*	++	+++	++	*	+++	+++	+	+++	+++	+++	+	+++	+++
Dulcitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Isodulcitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Salicin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

TABLE 5

Control tests

INGREDIENTS	STRAINS											
	B. typhi		*B. paratyphi* A		*B. paratyphi* B		Pneumococcus I		*Streptococci pyog.*		No germ	
Arabinose	−	−	+++	+++	+++	+++					−	−
Xylose	+++	+++	−	−	+++	+++					−	−
Glucose	+++	+++	+++	+++	+++	+++					−	−
Laevulose	+++	+++	+++	+++	+++	+++					−	−
Galactose	+++	+++	+++	+++	+++	+++					−	−
Mannose	+++	+++	+++	+++	+++	+++					−	−
Maltose	+++	+++	+++	+++	+++	+++					−	−
Lactose	−	−	−	−	−	−					−	−
Sucrose	−	−	−	−	−	−					−	−
Dextrin	+++	+	+++	+	+++	−					−	−
Amylum	−	−	−	−	−	−					−	−
Inulin	−	−	−	−	−	−	+	+++			−	−
Glycerin	−	+++	−	+++	−	++					−	−
Mannitol	+++	+++	+++	+++	+++	+++					−	−
Dulcitol	−	−	+++	+++	+++	+++					−	−
Isodulcitol	−	−	+	+++	+++	+++					−	−
Salicin	−	−	−	−	−	−			*	++	−	−

+++ indicates litmus color tones corresponding to pH $\leqq$ 5.5.

Control tubes for each sugar without inoculation assumed no change in color tone, but a slight condensation caused by evaporation took place.

EXPERIMENT II. REVERSION EXPERIMENTS ON THE LOST FERMENTATION OF SUCROSE BY "KOCH"

1. Passage through Fraenkel's solution containing sucrose

It has been variously reported that bacteria cultured in sugar containing media, adapt themselves to environment and acquire the power of fermenting the corresponding sugar.

Wortman (1882) observed that germs could produce the amylolytic ferment when they had been cultured in a medium containing no carbon source except starch. Hiss (1904) describes a bacillus of the dysentery group which acquired the power to ferment maltose after being grown for some time in a maltose medium. Twort (1907) found that dysentery bacilli which did not normally ferment sucrose, did so after cultivation in a medium containing this ingredient, and by similar means the true Shiga-Kruse organism was induced to ferment lactose. In the same way he could train typhoid bacilli to ferment dulcitol in a short period, but to ferment lactose only after two years' training. Lunz (1910) insisted that he could induce dysentery bacilli to ferment sucrose, maltose and dextrin, by passage through 1 per cent sugar pepton water. Penfold (cited by Gurney-Dixon, 1919) induced *B. typhi* to ferment dulcitol, arabinose and isodulcitol in a shorter interval; he failed however to develop new fermenting powers towards lactose and sucrose with the same organism. Bradley (cited by Gurney-Dixon, 1919) repeated these experiments with success as regards dulcitol and arabinose. Schroeter and Gutjahr (1911) succeeded in inducing dysentery bacilli to split sucrose and maltose. Matsuo and Izawa (1920) used an inorganic medium, Sasaki's solution, with success to train the dysentery organism of Y type to ferment sucrose, lactose, maltose and dextrin.

The reports are full of theoretical interest, above all, the induction of sugar splitting power in a protein-free medium as performed by Matsuo and Izawa with all theoretical considera-

tions in mind. In a protein-free meager medium, bacteria will be forced to utilize the sugars contained there, as an energy source, and may adapt themselves to ferment it more readily.

To recover the lost ability to ferment sucrose of the strain "Koch," the author applied passage experiments in a protein-free medium, Fraenkel's solution.

Preparation of the medium employed. Fraenkel's solution was adjusted to pH = 7.0, sucrose (Kahlbaum, Berlin) was added to it up to 1 per cent and divided into two portions; suitable quantities of brom cresol purple and brom thymol blue solutions were added respectively to each portion; and then they were tubed and sterilized with intermittent method as usual.

Carrying out of the training. "Koch" was inoculated into two media, thus prepared. Transplantations were carried out at intervals of one week, respectively with the same two kinds of media as in the case of preceding generations. The passage was interrupted by an accident (earthquake) in the course of the tenth generation.

Results of the experiment. No sign of reacquired acidifying power was observed, in any of the generations but on the contrary, constant turning towards alkalinity was indicated by brom thymol blue. The indicators gradually faded in the course of the culture period.

Control tubes inoculated with "Inaba" and "Takahashi," cholera strains having the normal ability to ferment sucrose, of the usual strains, were acidified to pH = 5.6 or 5.4 in a day or two, as indicated by the brom cresol purple.

2. Animal passage

If the defect of the sucrose fermentation of "Koch" is due to degeneration, refreshing by an animal passage might bring the return of this ability.

"Koch," having sufficient virulence to kill guinea pigs weighing 200 grams with less than 1:10 loopful of germs, after the performance of eight passages through guinea pigs, was examined with Aronson's plates and sucrose litmus pepton water; no red colony or sign of reversion, however, resulted.

3. Agar and broth passage

From the first examination of sugar fermentation on January 27, 1922, until the last occasion of the same test carried out on June 25, 1923, numerous agar passages were performed with the strain "Koch." In spite of repeated agar transplantations the loss of sucrose splitting power was inheritable during this period covering seventeen months.

Twenty broth passages, transplanting twice daily, after the method described by György (1920), also showed no results.

SUMMARY AND DISCUSSION

1. Twenty-one selected strains of the *V. cholerae* of different ages and from various sources, behaved entirely unanimously toward twelve carbohydrates, four polyatomic alcohols and one glycoside.

Sub-grouping concerning the sugar fermenting activities, as seen with other bacteria species, has been proved not to be possible with cholera germs. The strains of three immunological varieties, the original and the varied types of Kabeshima and the middle type of the author, exhibit the same attitude in the fermentation of various sugars.

2. Eight non-choleraic spirilla of different ages and from various sources display almost the same reaction towards carbohydrates and polyatomic alcohols as the strains of *V. cholerae*. Only a few strains reveal one or two disagreements in certain sugars. Hence the fermentation of sugars, as examined by the author, can not serve as a means to differentiate the *V. cholerae* from the non-choleraic spirilla.

The selective media for *V. cholerae* based upon the decomposition of sucrose and amylum, devised by Bendick, Aronson and Kodama, have no capacity to discriminate the *V. cholerae* and the non-cholera spirilla.

3. *V. cholerae* as well as most of the non-choleraic spirilla strains uniformly ferment the carbohydrates and polyatomic alcohols: glucose, levulose, galactose, dextrin, amylum and mannitol. The fermentations of galactose and mannitol are estimated to be a little weaker.

They entirely fail to ferment the following sugars and alcohols: arabinose, xylose, inulin, dulcitol, isodulcitol (rhamnose) and salicin.

The various strains, decompose, in different degrees, lactose and glycerol.

4. *V. cholerae* as well as non-choleraic spirilla form acids, but no gas in the sugar fermentation, even in the case of other carbohydrates than those so far studied by other authors.

5. The fact that every strain of *V. cholerae* and almost all other spirilla ferment all kinds of hexoses, but not both pentoses, may be a question of theoretical interest.

Most carbohydrates and polyatomic alcohols are split intensively or not fermented at all, while lactose and glycerol are attacked in different degrees by various strains. This is also a noticeable phenomenon.

6. No strains of *V. cholerae* reveal the reversion from once attained intensive acidity to alkalinity in 1 per cent sugar pepton water, while with some other spirilla, the reversion occurred in certain tubes. The reversion may have been due to a lower physiological constant in such combination of sugars and strains, or before reaching the pH death point alkaline products from the albuminous substances may begin to overbalance the acids produced from sugars.

7. A strain of *V. cholerae-immobilis*, "Koch," forms a single exception in failing to ferment sucrose. As "Koch" is able however to ferment glucose and levulose, the loss of the ability to redden the sucrose media must be due to the lack of the invertase formation in this strain.

As will be noted in the following article by the author, "Koch" possesses all the characteristics of a cholera vibrio, except for its immobility and the lack of sucrose fermentation (Studies upon the Immobile Strains of *V. cholerae*).

8. The anomaly of fermentation of the "Koch" strain was proven to be inheritable for seventeen months and withstood all reversion experiments. It seems to be a permanent inheritable varied nature; in other words, this variation may be an example of transformation after Reichenbach or the permanent "Klon-Umbildung" of E. Lehmann.

9. In the application of selective media for *V. cholerae* based upon the sucrose fermentation, such as those invented by Bendick and Aronson, attention must be paid to strains like "Koch." As animal passage with "Koch" resulted in no reversion of the mentioned disability, it may fail to ferment sucrose, even if it attacks the human body. Moreover, this strain has moderate virulence against guinea pigs, and gains a distinct virulence increase by animal passage. Such a sucrose non-fermenting strain may perhaps be able to cause an epidemic.

REFERENCES

ARONSON, H. 1915 Deut. med. Wochenschr., **41**, 1027.

BENDICK, A. J. 1912 Centralbl. f. Bakt., **62**, 536.

BITTER, H. 1896 Arch. f. Hyg., **5**, 241.

CLARK, W. M. 1922 The Determination of Hydrogen Ions, Williams & Wilkins, Baltimore.

FERMI, C. 1890 Arch. f. Hyg., **10**, 1.

FERMI, C., AND MONTESANO, G. 1896 Centralbl. f. Bakt., II Abt., **1**, 1.

GORDON, M. H. 1905 Centralbl. f. Bakt., **42**, 5.

GURNEY-DIXON 1919 The Transmutation of Bacteria, Cambridge University Press.

GYÖRGY, P. 1920 Centralbl. f. Bakt., **84**, 321.

HISS 1904 Cited from Gurney-Dixon, The Transmutation of Bacteria, Cambridge University Press, 1919.

ITAKURA, T. 1923 Eisei-Densenbyo-Gaku-Kai Zasshi (Japanese), **18**, 264.

KABESHIMA, T. 1913 Jour. Bact. (Japanese), 517.

KABESHIMA, T. 1918a Jour. Microörg. (Japanese), **6**, 1.

KABESHIMA, T. 1918b Compt. rend. Soc. Biol., **81**, 618.

KODAMA, T. 1921 Eisei-Densenbyo-Gaku-Kai Zasshi (Japanese), **17**, 26.

KUPRIANOW, J. 1893 Arch. f. Hyg., **19**, 282.

LUNZ, R. O. 1910 Centralbl. f. Bakt., **56**, 28.

MATSUO, I., AND IZAWA, T. 1920 Kyoto med. Jour. (Japanese), **17**, 1254.

MÜLLER, F. 1899 Centralbl. f. Bakt., **26**, 51 and 801.

OYAMA, S. 1921 Eisei-Densenbyo-Gaku-Kai Zasshi (Japanese), **17**, 30.

SCHROETER AND GUTJAHR 1911 Centralbl. f. Bakt., **58**, 577.

SCLAVO 1892 Cited from Fuhrmann, "Vorlesungen über Bakterienenzyme," Fisher Jena, 1907.

TEAGUE AND TRAVIS 1916 Jour. Inf. Dis., **18**, 601.

TWORT, E. A. 1907 Centralbl. f. Bakt., Ref. **40**, 508.

VIOLLE, H. 1919 Le Cholera, Masson, Paris.

WORTMANN 1882 Quoted from Pringsheim, "Die Variabilitat niederer Organismen," Springer, Berlin, 1910.

YAMADA, T. 1919 Japanese Jour. Exper. Med. (Japanese), **3**, 85.

THE REDUCING PROPERTIES OF MICROÖRGANISMS WITH SPECIAL REFERENCE TO SELENIUM COMPOUNDS[1]

VICTOR E. LEVINE

Department of Biological Chemistry and Nutrition, School of Medicine, Creighton University, Omaha, Nebraska

Received for publication July 23, 1924

The reducing power possessed by bacteria was a characteristic early recognized. In 1843 Helmholtz observed in putrefactive mixtures the reduction of litmus to its leuco base. The first observers to make direct researches in reference to the reducing power of bacteria were, however, Cahen, Spina, and Rozahegyi. Later came the work of Petruscky, Würtz, Behring, Smith, Müller, Uffelmann, Grasser, Marpmann and Rothberger, all of whom used dyes as indicators. Besides dyes other organic compounds may undergo reduction. Amino acids are reduced by anaerobes with the formation of saturated fatty acid and ammonia. Gayon and Dubourge observed the conversion of fructose into mannitol, Labbé, of oxyhemoglobin into hemoglobin, and Alsberg, of oxyhemocyanin into hemocyanin. The formation in human feces of coprosterol from cholesterol and of stercorcin from bilirubin is believed to be the result of bacterial reduction.

Inorganic compounds also suffer reduction. The reduction of ammonium molybdate has been reported by Capaldi and Proskauer and also by Levine and Jahr, and ferric compounds have been transformed into ferrous compounds by Poehl. That nitrates in the soil are capable of being converted to nitrites was first observed in 1862 by Goppelsröder. Meusel attributed this

[1] This work was partly done in the biochemical laboratory of the College of Physicians and Surgeons, Columbia University, and partly at the Museum of Natural History, New York City.

phenomenon to bacterial action. Sulfates are reduced to hydrogen sulfid, according to Stockvis and Saltet, Kochmann, Van Delden and others. Not only sulfates but also sulfites, tetrathionates, pentathionates, sulfur in organic complexes such as peptone, cystine and thiourea yield hydrogen sulfid. Free sulfur is also converted into hydrogen sulfid, but selenium, tellurium, arsenic and antimony do not yield the corresponding hydrogen compounds.

Bacteria are also capable of carrying on oxidations. The existence of autotrophic microörganisms, capable of deriving energy through the oxidation of purely inorganic substances has become an established fact. The nitrate and nitrite bacteria, the sulfur bacteria and the iron bacteria are examples of such types. Recently Lipman and Waksman have added another group of organisms to the already known autotrophic forms. These organisms secure energy from the oxidation of selenium. Lipman and Waksman mixed a small quantity of selenium with the soil and observed an increase in the acidity of the latter. They transferred some of this soil to a culture medium consisting of inorganic materials with elemental selenium as the only source of energy. The medium became cloudy within a few days and there appeared in it a minute rod-shaped bacterium capable of oxidizing selenium to selenic acid, just as the sulfur bacterium is capable of oxidizing elemental sulfur to sulfuric acid.

Whether selenium or tellurium could replace sulfur in the metabolism of sulfur bacteria was the object of an investigation of W. Brenner. One species, which he isolated from marine mud, *Micrococcus selenicus*, made good growth in sodium selenid without sodium selenite. The latter could be replaced by other substances such as sodium selenate, sodium thiosulfate, indigocarmin and litmus but not by potassium tellurite, potassium sulfate, potassium nitrate or atmospheric oxygen. Another species isolated from marine mud grew well on a substance containing sodium selenite, but a third species, *Thiobacillus thioparus*, gave entirely negative results. Free selenium was found in the bacterial cell when grown on selenite medium. An odor

similar to that emanating from molds, when grown with sodium selenite was observed in the presence of sodium selenid, but not in the presence of sodium selenite alone.

That bacteria reduced certain compounds of selenium was first mentioned by Japha in 1842. To quote from his dissertation, "*Bacillus ferreus* urinae immissus post aliquod tempus colorae fusco rubro tinctus est, paulatimque selenium purum floccis rubiis in illo praecipitatum." He utilized the reducing power of *Bacillus ferreus* to determine the presence of selenium dioxid. Not finding any reduced selenium by this biochemical method as well as by several purely chemical procedures, he concluded that the selenium was not eliminated in the urine.

In 1890 Chabrié and Lapicque performed a single experiment which showed that a 0.2 per cent solution of selenious acid prevented the putrefaction of bouillon. In smaller concentration, however, putrefaction did occur. At the same time the selenious acid was reduced and colonies, which alone were colored red, were observed in the liquid. These authors attributed the reduction of the selenium compound to the activity of the microorganisms in the bouillon.

In 1900 Scheuerlen grew cultures of *Bacillus anthracis* on a medium containing either sodium selenite or sodium tellurite. These he added to the medium because of a desire to grow the bacillus in pure culture and in the absence of atmospheric oxygen, which might bring with it contaminating organisms. He had hopes that this sodium or tellurium salt, which is easily reduced, would act similarly to the loosely bound oxygen in oxyhemoglobin and would be almost as favorable to the growth of the anthrax bacillus as blood itself. He found, however, that although these salts were reduced to red selenium or dark gray tellurium, growth nevertheless suffered retardation. Continuing his observations, he found that not only *Bacillus anthracis* but also all of the growing bacteria he examined were colored in the presence of sodium selenite or tellurite. Pigmentation occurred in the bacterial cell, but the nutrient medium itself was free from color.

The results of Scheuerlen led directly to the more extensive

work of his pupil, Klett, who made a study of twenty-seven species of bacteria and molds under the influence of sodium tellurite, sodium selenite and sodium selenate. He also reported that the tellurite and the selenite were reduced by living organisms. On agar streak cultures, colonies grew in gray-black streaks in the presence of the tellurium compound. Reduced selenium accumulated for the most part in the water of condensation.

In general Klett found that sodium selenite or tellurite did not favor the growth of bacteria and that the intensity of the reduction was proportional to the intensity of growth. These two salts had no effect on the virulence of bacteria. Aerobic bacteria grown under anaerobic conditions could not thrive on the oxygen set free in the course of the reduction of sodium selenite. Klett also observed that, when the nutrient medium contained glucose, reduction took place at 37°C. even in the control tubes. He was obliged therefore to allow growth in such media to proceed at room temperature.

Klett believed that reduction took place within but not without the bacterial cell. He observed that reduction followed the line of growth, the red or gray color not being diffused throughout the medium. A microscopic examination of bacterial bodies revealed the presence of yellowish or grayish particles within the cell. Sodium selenate, like sodium sulfate or sodium phosphate showed no inhibition of growth, nor was there any evidence of reduction of the selenate to brick red selenium.

Gosio in 1904 took up the problem of the bacterial reduction of selenium and tellurium salts in the hope of obtaining a method for determining the sterility of sera, culture media, body fluids, foods and other biological substances. With this end in view he examined 181 organisms grown upon selenite or tellurite media. He came to the conclusion that sodium selenite was more easily reduced than the tellurite by substances other than bacteria. He therefore preferred the tellurite and employed this salt for most of his experiments. King and Davis have recently confirmed Gosio in proving the value of tellurite medium as an indicator of microbial life.

According to Gosio tellurite reduction was accelerated in a milk medium or in a medium containing glucose, sucrose or lactose. Glucose seemed to be the best accelerator. He attempted to explain these findings on the basis of the supposition that lactic acid was produced, which, interacting with the potassium tellurite gave rise to the more easily decomposed tellurous acid. The limit of sensitiveness in a sugar-free medium was 1:20,000, whereas in a sugar medium, reduction was noticeable when the latter contained one part of the tellurite in 75,000, or even 100,000 parts of the nutrient substance. Levine has found that at 30°C. to 37.5°C. or over and under sterile conditions, glucose, lactose, and lactic acid themselves cause reduction of sodium selenite. The accelerated reduction, noted by Gosio, was really due to action of these reducing substances in the nutrient medium.

Gloger in 1906 repeated some of the work of Gosio. He agreed with the latter that the presence of glucose accelerated reduction and stated that the kind of medium used was a very important factor in determining bacterial reduction. He, however, did not make any effort to determine what chemical substances present in the nutrient medium would interfere. He formulated a relationship between the production of hydrogen sulfid and the reduction of potassium tellurite. Finding that *B. tuberculosis-hominis*, *B. pseudotuberculosis* Pfeiffer, *B. acidi-lactici*, *Spirillum rubrum*, *B. diphtheriae* and *B. pseudodiphtheriae* did not reduce, he put forth the proposition that these organisms were not active because they evolved only traces of hydrogen sulfid or none at all, the hydrogen sulfid itself having the power of causing the reduction of selenite or tellurite. W. Kruse in his "Allegemeine Mikrobiologie" published in 1910 stated that anaerobes did not reduce, and that small amounts of selenite or tellurite impeded their growth, while small quantities of sulfite acted favorably. He also mentioned the fact that *B. tuberculosis-hominis*, *B. pseudotuberculosis*, *B. diphtheriae*, *B. pseudodiphtheriae*, and *B. acidi-lactici* did not reduce. He, too, gave hydrogen sulfid as the cause of reduction.

In the work here reported the effect of various selenium com-

pounds upon the growth and upon the reducing power of some of the important groups of bacteria was studied. Experiments were also undertaken to prove or disprove the statement that anaerobes did not reduce, and to ascertain the validity of Gloger's conclusion that hydrogen sulfid was the cause of reduction. Still other experiments were carried out to determine the nature of bacterial reductase. The organisms studied were obtained from Prof. C.-E. A. Winslow's valuable collection in the American Museum of Natural History, New York City.

EFFECT OF SELENIUM COMPOUNDS UPON THE GROWTH OF BACTERIA

The effect on growth was studied with a view towards discovering any relation between growth and reduction. Selenium dioxid, selenic acid, sodium selenite, sodium selenate, and potassium selenocyanid were the compounds employed. Colloidal selenium was not included, but it has recently been shown by Henry Crooke that it has no germicidal action.

The organisms tested were *Bacillus coli-communis* and *Streptococcus pyogenes-aureus*. The medium used was liquid broth, acid in reaction (pH 6.8). The nutrient media were made up with increasing amounts of the selenium salts or acids. The concentrations employed for sodium selenate were 0.15, 0.28, 0.40, 0.50, 0.60, 0.68, 0.77, 0.83, 0.90, 0.96 per cent. These figures are on the basis of crystalline sodium selenate ($Na_2SeO_4 \cdot 10H_2O$). For selenium dioxid (SeO_2), selenic acid (H_2SeO_4), sodium selenite (Na_2SeO_3) and potassium selenocyanid (KCNSe) lower dilutions were used: 0.06, 0.11, 0.16, 0.20, 0.24, 0.27, 0.30, 0.33, 0.36, and 0.38 per cent. The tubes were examined after twenty-four, forty-eight and seventy-two hours.

Briefly stated, the results were as follows: At the end of twenty-four hours only the tubes of the lowest concentration showed growth but after seventy-two hours all of the tubes gave evidence of growth. *Streptococcus pyogenes-aureus* proved more sensitive to the selenium compounds tested than did *Bacillus coli*. Thus at the end of the first day *B. coli* showed growth in the tubes containing 0.15 to 0.95 per cent sodium selenate, while

the streptococcus gave growth in the 0.15, 0.28 and 0.40 per cent tubes only. In degree of toxicity the compounds may be arranged in the following series of diminishing effect: selenium dioxid (selenious acid), selenic acid, sodium selenite, sodium selenate, potassium selenocyanid. This order agrees well with that found by Levine in a study of the toxic effect of selenium compounds upon yeast, plants and animals.

Selenium dioxid and selenic acid in concentrations as low as 0.06 per cent exercised a marked inhibitory effect. Sodium selenite did not retard growth as much as selenious acid. Sodium selenate and potassium selenocyanid in the very low concentrations had slight effect on the growth of the organisms tested. It is interesting to mention that Němec and Kás found that extremely small amounts of sodium selenate increase the development of certain types of molds, even in the presence in the culture medium of zinc and manganese compounds.

A very interesting feature of the experiments was the formation in cultures containing selenious acid, sodium selenite, and selenic acid of free brick-red selenium, which in part precipitated and in part suffered colloidal dispersion in the liquid medium. Reduction took place only where growth occurred and the profuseness of reduction paralleled that of growth. Selenic acid was reduced more slowly than selenious acid. This fact seems to indicate that reduction takes place in two stages. At first the selenic acid is reduced to selenious acid and this is further reduced to free selenium.

Sodium selenate was not reduced to free selenium. The formation from potassium selenocyanid of free selenium only occurred as a result of the production by the organism of acid metabolites. The formation of free selenium from this compound cannot be looked upon as a vital phenomenon. Levine and Ahana have shown that acid solutions have the power to decompose it with the formation of free selenium. The reaction depends upon the hydrogen ion concentration. They have proposed the use of potassium selenocyanid as a qualitative test for free acidity in the gastric contents and have found it as efficient as Töpfer's reagent. While growth after

TABLE 1

	SELENIUM COMPOUNDS	STRENGTH OF SOLUTION	ORGANISM	REMARKS
1	Selenium dioxid.....	1:4,000	*B. coli*	At the end of twenty-four hours, colonies only pinkish. The rest of medium unchanged in color. Number of colonies not so many as in the control plates. Control containing selenium dioxid, but no bacteria, showed no evidence of reduction. When again examined at the end of six days the pink-red spots were larger in diameter and brick-red in tint
2	Selenium dioxid.....	1:2,000	*B. coli*	Slight growth, and slight reduction. When examined again at the end of six days profuse pigmentation of the colonies noticed. There seemed to be greater reduction than in plate 1 and also better growth
3	Selenium dioxid.....	1:4,000	*Strep. pyog.*	No microscopic evidence of bacteria at end of first day. Slight pigmentation of the colonies observed after six days
4	Selenium dioxid.....	1:2,000	*Strep. pyog.*	No growth and no reduction. At the end of six days fewer colonies and slighter reduction than in plate 3
5	Selenic acid.....	1:4,000	*B. coli*	Pink colonies at the end of first day. Colonies more in number than in plate 7. At the end of six days localized reduction profused. Good growth
6	Selenic acid.....	1:2,000	*B. coli*	Few pink colonies. At the end of six days reduction profuse and growth good
7	Selenic acid.....	1:4,000	*Strep. pyog.*	Few pink colonies at end of first day. At end of a few days number of colonies slight and reduction therefore not profuse
8	Selenic acid.....	1:2,000	*Strep. pyog.*	At the end of first day no growth and no reduction. At end of six days very few slightly pigmented colonies

9	Sodium selenite.....	1:2,000	*B. coli*	Growth less than in control. Few pink colonies. At end of six days profuse pigmentation of colonies
10	Sodium selenite.....	1:1,000	*B. coli*	Growth less than in plate 9. Reduction also less marked. At the end of six days more marked reduction.
11	Sodium selenite.....	1:2,000	*Strep. pyog.*	Growth slight. Small pink-red colonies noticeable. At the end of six days very marked reduction
12	Sodium selenite.....	1:1,000	*Strep. pyog.*	Growth very slight. Very few pink-red colonies. Profuse localized reduction at end of six days
13	Sodium selenate.....	1:1,000	*B. coli*	Growth as good as in control. No reduction observed even at the end of six days
14	Sodium selenate.....	1:500	*B. coli*	Growth as good as in control. No reduction to be found after six days
15	Sodium selenate.....	1:1,000	*Strep. pyog.*	Growth as good as in control. No reduction
16	Sodium selenate.....	1:500	*Strep. pyog.*	Growth as good as in control. No reduction
17	Potassium selenocyanid.....	1:2,000	*B. coli*	Excellent growth as good as in control but no pigmentation. At end of six days colonies pigmented
18	Potassium selenocyanid.....	1:1,000	*B. coli*	Growth but no reduction. When examined at end of six days pinkish-red colonies observed
19	Potassium selenocyanid.....	1:2,000	*Strep. pyog.*	Growth but no reduction. At the end of six days colonies reduced
20	Potassium selenocyanid.....	1:1,000	*Strep. pyog.*	Growth not as good as in control. Colonies pigmented. More abundant pigmentation at the end of six days

the first day occurred in all the *B. coli* and *streptococcus* tubes, none showed reddening. On the second day as acidity developed some tubes showed reddening and the number of tubes giving free selenium increased on the third day.

LOCALIZATION OF SELENIUM IN THE CULTURE MEDIUM

In order to gain a better idea as to the localization of the red deposits it was thought advisable to employ a solid nutrient medium such as peptone agar. All the solid media used in this series of experiments and in experiments following were rendered sugar-free by inoculation with *B. coli* and subsequent sterilization. To each petri dish were added peptone agar, 0.05 cc. of broth containing a heavy growth of *Bacillus coli* or *Streptococcus pyogenes*, and also a definite amount of the selenium compound. The total volume was made up to ten cubic centimeters. The culture plates were then kept in the incubator at 37.5°C.

The results are tabulated in table 1.

It is seen from these experiments that reduction followed the path of growth. Colonies alone were colored brick-red. The barren regions of the medium remained unchanged in color.

Tellurium dioxid in concentrations of 1:10,000 was also reduced by *B. coli*. The colonies alone showed up in grayish-white circles, in the center of which dark gray metallic tellurium spots were especially noticeable.

IS BACTERIAL REDUCTION CAUSED BY HYDROGEN SULFID?

Desiring to confirm the statement made by Gloger that only organisms that produce sulfuretted hydrogen were able to cause reduction, the author repeated Gloger's experiments using, however, selenium dioxid and sodium selenite instead of potassium tellurite.

On growing the organisms used by Gloger, *B. acidi-lactici*, *B. diphtheriae*, *B. pseudodiphtheriae*, in a sugar-free medium to which had been added five or six drops of 1 per cent solution of neutralized sodium selenite, faint reduction was found to have occurred at the end of twenty-four hours along the path of the

stab of the cultures of *B. pseudodiphtheriae*. Cultures of *B. coli*, serving as controls, showed remarkable profuseness of reduction. At the end of two weeks, pigmentation was not found in the *B. diphtheriae* and *B. acidi-lactici* cultures, although control cultures, which had indicated reduction at the end of one day, showed greater reduction at the end of the fortnight.

Believing that reduction did not take place because of the high concentration of sodium selenite, the experiments with *B. acidi-lactici* and *B. diphtheriae* were repeated. This time only two drops or 0.1 cc. of a 1 per cent solution of selenium dioxid or 0.1 cc. of a 0.2 per cent solution of sodium selenite were used. At the end of twenty-four hours the two organisms showed good reduction along the line of growth, in both the selenium dioxid and in the sodium selenite media.

Tabulating the results of different workers, we find the following condition in respect to Gloger's statement:

NAME OF ORGANISM	H_2S PRODUCTION	GOSIO 1905— Na_2TeO_3	GLOGER 1906— K_2TeO_3	LEVINE 1913— Na_2SeO_3	KLIGLER 1913— SeO_2	BELFANTI 1914— Na_2TeO_3
B. tuberculosis-hominis....		Faint reduction	Negative	Reduction		Reduction
B. tuberculosis-avium... ..		Faint reduction	Negative			Reduction
B. diphtheriae.....	Traces	Faint reduction	Negative	Reduction	Reduction	
B. pseudotuberculosis (Pfeiffer).....		No reduction	Negative			
B. pseudodiphtheriae.....	Traces	Faint reduction	Negative	Reduction	Reduction	
B. acidi-lactici.....		Strong reduction	Negative	Strong reduction		
Spirillum rubrum.....		Not tried	Negative	Not tried		

Kligler, in looking for a rapid and convenient method of differentiating *B. diphtheriae* from *B. pseudodiphtheriae*, attempted to grow these organisms on media containing one part of selenium dioxid in fifty thousand. He found reduction in both organisms. When grown on slant cultures, those organisms on the surface of the slants were usually unpigmented, but reductions were always observed within the medium, the reduced selenium accumulating as a rule in the water of condensation.

REDUCTION OF ANAEROBES

Klett found that sodium selenite so markedly arrested the growth of the anaerobes, of malignant edema and symptomatic anthrax, that no reduction was observed. They, however, reduced sodium tellurite and in the presence of this salt growth continued vigorously. Gosio in his first paper dealing with selenite and tellurite reduction found that the bacilli of oedema, tetanus and symptomatic anthrax hardly gave any growth in his special media and manifested no reducing power. He used very high concentrations, a few drops of a 20 per cent solution in each tube. W. Kruse in his text book stated that while selenites and tellurites are reduced by aerobic bacteria no such action can be induced by anaerobic cultures. It seems important to ascertain these points, for if selenium is to be used as an index of bacterial contamination, growth of anaerobes would pass unnoticed.

In order to put these facts to the test, there were made some preliminary experiments with the organisms of symptomatic anthrax, oedema and tetanus. It was found that on the addition of five or six drops of 2 per cent sodium selenite to a sugar-free medium no growth was obtained. It seems that the concentration of selenite was too great and the experiment was therefore repeated, using 0.15 cc. of a 1 per cent sodium selenite. Other experiments were tried with 0.15 cc. of 1 per cent potassium selenocyanid and 0.1 cc. of a 0.5 per cent selenium dioxid. At the end of twenty-four hours the potassium selenocyanid did not inhibit the growth of these organisms, and gas formation had taken place in the tetanus tube. No red pigmentation was

TABLE 2

Reduction of sodium selenite: anaerobes

NAME OF ORGANISM	CONCENTRATIONS											
	1:100,000			1:50,000			1:25,000			1:10,000		
	Reduction at end of			Reduction at end of			Reduction at end of			Reduction at end of		
	24 hours	48 hours	96 hours	24 hours	48 hours	96 hours	24 hours	48 hours	96 hours	24 hours	48 hours	96 hours
B. welchii, 521*	+	−	−	+	+	+	+	+	+	+	++	++
B. welchii, 500	+	+	−	+	+	+	+	+	+	++	++	++
B. welchii, 20	+	−	−	+	+	+	+	+	+	++	++	++
B. welchii, 13	+	+	−	+	++	++	+	+	+	−	−	−
B. feseri, 48	−	−	−	+	+	+	−	+	+	+	+	+
B. feseri, 53	+	−	−	+	+	+	−	+	+	+	+	++
B. tetani, 274	+	−	−	+	+	+	−	++	+	+	+	+
B. tetani, 1	+	−	−	+	+	++	+	+	+	−	−	−
B. sporogenes, 120	+	−	−	+	+	+	+	+	+	++	++	++
B. sporogenes, 138	+	−	−	+	+	+	++	++	+	++	++	++
B. sporogenes, 425	−	−	−	+	+	+	+	+	+	++	++	++
B. oedematis, 421	+	−	−	+	++	++	−	+	+	−	−	−
B. oedematis maligni, 558	−	+	+	+	+	++	+	+	+	−	−	−
B. oedematis maligni, 485	+	−	−	+	+	++	−	+	+	−	+	+
B. botulinus, 595	+	−	−	+	+	+	+	+	+	+	+	+
B. putrificus, 459	−	+	−	+	+	+	−	++	+	−	−	−

* The number after the name of the bacillus represents the number of the organism in the bacteriological collection at the Museum of Natural History.

noted in any of the selenocyanid tubes. In the oedema culture containing sodium selenite and in the one containing selenium dioxid there was very slight growth. In both tubes reduction alone the line of meager growth had taken place. In the tetanus tubes there was no growth and no reduction in the selenium dioxid or in the sodium selenite medium. Selenium dioxid and sodium selenite tubes inoculated with symptomatic anthrax displayed no growth and consequently no reduction.

At the end of the second day the reduction observed with the oedema bacillus was more profuse. There was greater growth in the sodium selenite tube. With the tetanus organism, reduction was noticeable in the selenium dioxid tube after two days, but no growth and no reduction in the sodium selenite tube. After the fifth day slight reduction was observed in the sodium selenite tube inoculated with symptomatic anthrax. After eight days the selenocyanid showed no free selenium formation.

It was considered probable that the selenocyanid in greater amounts would form brick-red selenium along the path of growth. *B. coli*, and the bacilli of symptomatic anthrax, oedema and tetanus were grown in a potassium selenocyanid medium containing 1 per cent lactose and also in a sugar-free medium. To each tube was added either 0.5 or 1 cc. of the selenocyanid. It was noted that the presence of these relatively large amounts of potassium selenocyanid caused a diminution in the growth of the anaerobic organisms and in the volume of the gas liberated. There occurred less growth in the tubes containing the larger amounts of the salt. No pigmentation, however, was observed at the end of the week, either in the sugar-free or in the sugar-containing medium; *B. coli*, however, showed reduction along the stab in the lactose medium containing 1 cc. of potassium selenocyanid. A sugar-free medium inoculated with *B. coli*, gave evidence of reduction at the end of three days. Evidently the presence of lactose favored acid production by *B. coli* and therefore acid decomposition of the selenium compound. When the tubes were allowed to stand the selenium tint diffused gradually through the medium.

A more systematic study of the anaerobes was determined

upon. Having found that the concentration of the selenium compounds was an important factor in growth and in reduction, sodium selenite was added to the medium employed in the following concentrations: 1:100,000; 1:50,000; 1:25,000; and 1:10,000.

Fifteen anaerobes, including *B. Welchii*, *B. tetani*, *B. chauvoei*, *B. putrificans*, *B. oedematis* and *B. botulinus* were tested in ordinary agar stabs. The tubes were inverted in alkaline pyrogallol solution and incubated, the results being recorded after one, two, three and four days respectively. An abundance of both growth and reduction was noted. As in the other experiments the intensity of reduction was a function of luxuriancy of growth. In all instances reduction was obtained along the line of growth only.

We find here a very striking corroboration of the fact long reported by Cahen, Smith, Kitasato and Weyl that anaerobes reduce as a result of the biogenic activity of the cell. These investigators, however, worked with dyes and the relation of the cell to the reducing process was not as strikingly brought out as in the case of the selenium compounds. Here reduction is localized and seems intimately related to the growth of the organism, while the presence of the free chocolate-red or brick-red selenium granules in the cell points strongly to the fact that the reduction is an intracellular phenomenon. The anaerobic bacteria evidently utilize oxygen for their metabolic processes just as the anaerobes do, and it is only oxygen in the free state that is inimical to the life of the organism.

No appreciable inhibition of growth was observed except in some cases in concentrations of 1:10,000. Reduction in dilutions of 1:100,000 was found to have taken place with most strains within forty-eight hours. At the end of two days the red selenium streak following the path of growth began to disappear and at the end of three days no sign of free selenium was visible except in the case of one strain of *B. oedematis-maligni* and *B. putrificans*. The higher selenite concentrations showed excellent reduction but there was less tendency for the precipitated selenium to disappear. At the end of three months

the brick-red selenium had completely disappeared in all the culture tubes except the ones containing sodium selenite in the proportion of 1:10,000. For practical purposes, concentrations of 1:25,000 give the best results, as all the strains tried reduced within twenty-four to forty-eight hours, while in 1:10,000 selenite agar, one strain of *B. Welchii*, one strain of *B. tetani*, *B. oedematis* and *B. oedematis-maligni* failed to reduce even at the end of four days.

REDUCTIONS WITH THE DIPHTHERIA GROUP

Gloger reported failure of *B. diphtheriae* and *B. pseudodiphtheriae* to reduce. This was probably due to the high concentration of selenium compound used. Joachimoglu and Hirose report that the following concentrations will kill diphtheria; 1:1160 for sodium selenite; 1:420 for sodium tellurite; 1:666 for selenate; 1:125 for tellurate. They employed a culture medium containing 4 per cent sugar. Conradi, on the other hand, made use of the reducing action of *B. diphtheriae* on tellurium salts for the isolation of these organisms. It seemed likely, therefore, that a more thorough study of the reducing powers of the different members of this group might reveal characteristics that would be of value in differentiating them from one another.

Four strains of *B. diphtheriae*, seven strains of pseudodiphtheriae and three strains of diphtheroid organisms from Hodgkin's disease were used. These were grown on agar slants containing 0.0001, 0.00004, 0.000001 gms. of sodium selenite or selenium dioxid, in 1000 grams of agar. Observations were made after five hours, twenty-four hours, four days and ten days. The nature of the growth and the intensity of the reduction were recorded.

In general, reduction was obtained with all the strains, the best reaction appearing in the 1:10,000 dilution of sodium selenite and in the 1:25,000 of selenium dioxid. It is evident that the concentration of the selenium reagent is the important factor in determining the course of reaction. It is also interesting to note in this connection that while reduction was obtained in dilutions as high as 1:100,000 and in some cases even in

TABLE 3

Selenium dioxid: diphtheriae group

Growth and reduction at end of five hours (*A*), twenty-four hours (*B*), four days (*C*), ten days (*D*)

NAME OF ORGANISM	CONCENTRATIONS																											
	1:200,000						1:100,000						1:50,000						1:25,000						1:10,000			
	A		B		C	D	A		B		C	D	A		B		C	D	A		B		C	D	A		B		C	D
	Reduction	Growth	Reduction	Growth	Reduction	Reduction	Reduction	Growth	Reduction	Growth	Reduction	Reduction	Reduction	Growth	Reduction	Growth	Reduction	Reduction	Reduction	Growth	Reduction	Growth	Reduction	Reduction	Reduction	Growth	Reduction	Growth	Reduction	Reduction
B. diphtheriae, 61	−	+	−	++	−	−	+	+	+	++	++	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. diphtheriae, 96	−	+	−	++	−	−	+	+	+	+	++	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+
B. diphtheriae, 97	−	+	−	++	−	−	+	+	+	+	++	++	++	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+
B. pseudodiphtheriae, 2	−	+	−	−	+	+	+	+	+	+	++	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	++
B. pseudodiphtheriae, 99	−	−	+	+	+	−	+	−	+	+	++	+	+	+	+	+	+	+	+	+	+	+	++	+	−	−	−	−	+	++
B. pseudodiphtheriae, 100	−	+	−	++	−	+	+	+	+	++	++	+	+	+	+	+	+	+	+	+	+	+	+	++	+	+	+	+	++	++
B. pseudodiphtheriae, 494	−	−	−	++	−	?	+	+	+	++	++	+	+	++	+	+	+	+	+	++	+	+	+	+	−	−	+	+	++	++
B. pseudodiphtheriae, 495	−	+	−	++	+	?	+	+	+	++	++	+	+	++	+	++	+	+	+	++	+	++	+	+	+	+	+	+	++	++
B. Hodgkini, 640	−	+	+	++	+	−	−	+	+	++	++	+	−	+	+	+	+	+	−	++	+	+	++	+	−	−	++	+	++	++
B. Hodgkini, 641	−	+	−	++	−	−	+	+	++	+	++	+	+	+	+	+	+	+	−	++	+	++	++	+	−	−	++	+	++	++
B. Hodgkini, 642	−	+	−	++	−	−	−	+	−	++	++	−	−	−	+	+	+	+	−	−	−	−	+	+	−	−	−	−	+	+
B. Hoffmani, 21	−	+	−	++	−	−	−	+	+	+	++	+	+	++	++	+	+	+	−	−	−	−	−	+	−	−	−	−	−	+
B. Hoagi, 497	−	+	+	++	−	−	−	+	+	++	++	+	+	+	+	+	+	+	+	+	+	+	++	+	−	−	+	+	++	++
B. serosis, 570	−	+	−	++	+	−	−	+	+	++	++	+	+	++	+	+	+	+	+	+	++	+	++	+	−	−	+	+	+	+

* The number after the name of the bacillus represents the number of the organism in the bacteriological collection at the Museum of Natural History.

1:200,000 the red color due to the deposition of selenium gradually faded away and disappeared completely after a few days. In the higher concentrations the fading was not as marked and the intensity of the color was not appreciably diminished after a month.

Gosio, Maassen and also Rosenheim have proven the formation of a volatile organic selenid or tellurid when microörganisms are grown in cultures containing compounds of selenium. Maassen showed that the alkyl selenid or tellurid produced by microorganisms was ethyl selenid or tellurid, while Hofmeister working with tissues of higher animals found the alkyl to be methyl. The fading, which was also observed with anaerobes, is probably due to volatilization of the selenium in the form of an organic compound.

Reduction is intimately associated with vigor of growth. Too high a concentration inhibits growth and gives diminished reduction. Too low a concentration, on the other hand, does not inhibit growth and may give no evidence of reduction; apparently it has no effect on the bacterial cell since the latter does not avail itself of the oxygen in this compound, as indicated by the absence of reduction. The diphtheria bacilli are more sensitive to the toxic action of the selenium compounds than are the diphtheroids. A marked inhibition is observed in the growth of the former in a dilution of 1:10,000 of the selenium dioxid, while the latter are affected but little by that concentration.

The abundance of oxygen likewise influences the intensity of reduction. Thus but little reduction is obtained on the upper part of the streak, while the reduction is more marked in the water of condensation and still more evident in the stab. Apparently these cells utilize the combined oxygen only when the oxygen pressure of the medium falls below a certain point.

Reduction of selenite and selenium dioxid is an intracellular phenomenon. The reduction always follows the line of growth in the stab culture. On streak growths only the discrete colonies (or the entire growth if the growth is confluent) are colored red while the rest of the medium is not colored. Reduction is al-

ways more intense in the center of the colony and fades away toward the edge. A microscopic examination reveals the red granules of elemental selenium deposited within the cell.

Aside from the difference in sensitiveness to the toxic action of the selenium dioxid, the diphtheria and pseudodiphtheria bacilli also show very distinct differences in the character of their growth on the streak. This characteristic difference most likely results from the inhibitive action of the selenium dioxid. The diphtheria bacilli grow in the form of small discrete colonies, of brick-red color (due to a deposition of selenium), while the pseudodiphtheria bacilli grow in larger colonies or in a continuous streak along the line of inoculation.

EXPERIMENTS WITH THE COLON-TYPHOID GROUP

All the members of the colon-typhoid group were tested by Kligler, Greenberg and the author, in a manner similar to that employed in the study of the diphtheria organisms with the exception that only one dilution (1:10,000) of sodium selenite was used. The cultures were streaked on agar slants containing the selenite and observations made after twenty-four hours, forty-eight and ninety-six hours, respectively. Forty-five colon-organisms, ten *dysenteriae* and twenty *B. typhi* were tested.

In brief, the results were as follows: After twenty-four hours all organisms gave distinct reduction except the *B. dysenteriae*, *paratyphi* A and the fowl cholera bacilli.[2] The coli, para-coli and enteritidis types and some *B. typhi* gave strongest reduction, the typhi and paratyphi B. followed closely. That *B. coli* is a better reducer than *B. typhi* has also been demonstrated with nitrate (Dieudonné, Lunkevicz), and with ammonium molybdate (Capaldi, Proskauer). After forty-eight hours *B. cholerae* showed reduction, while the dysenteriae and paratyphi A still failed to show any or but faint traces of reduction. Practically the same result was obtained after ninety-six hours. These results are significant in that they strengthen the view that the colon para-colon enteridis group is closely associated,

[2] With lower concentration of sodium selenite Gosio obtained reduction with dysentery and paratyphoid organisms.

that *B. typhi* and *B. paratyphi* B are more intimately related, while the *paratyphi* A and *dysenteriae* form a third division of the family.

Handel and Theodorascu observed that most strains of *B. coli* were more inhibited in their growth by sodium selenite than were typhoid bacilli; Guth was able to confirm this and found that the selectivity for the latter organism was increased by an alkaline reaction. The optimum concentration of the selenium salt he found to be 0.15 per cent. A comparison with malachite green and Endo agar showed the superiority of selenium agar as a culture medium for the selective growth of typhoid bacilli. As a practical medium Guth used selenium-agar to which was added 0.1 per cent crystal violet.

SELENIUM COMPOUNDS AS INDICATORS OF MICROBIAL LIFE

The use of dyes, such as methylene blue, as indicators of biologic reducing processes is by no means ideal. Methylene blue is very easily reduced by a great many compounds which may happen to be in the culture medium. Hasse has shown that among the amino acids, glycine is the most potent in bringing about the reduction of methylene blue. The leuco form of the dye, moreover, is very easily re-oxidized to methylene blue by exposure to air or by shaking. This fact can be readily illustrated by the following experiment. Leuco-methylene blue can be prepared by boiling with zinc dust an aqueous solution of the dye made alkaline with sodium hydroxide. The colorless liquid which results becomes blue on the addition of tap water or on slight shaking.

Selenium compounds as indicators are of greater value. The reduction is localized and for that reason cannot be ascribed to bacterial metabolites. Furthermore, reduction of the selenium compound is an irreversible reaction. The precipitated selenium shows no tendency to reoxidize.

In order to serve as indicators of microbial life selenium dioxid or sodium selenite should be reduced by all microörganisms. Those, which at first evinced no such power, did not reduce owing to the presence in the medium of too high a concentration of the

selenium compound. It will be remembered that selenium dioxid or sodium selenite in too great amounts can prevent growth entirely. Without growth there is no reduction, for the intensity of the latter process is directly proportional to that of the former. The result of retardation in growth depends not only upon the concentration of the selenium compound but also upon the nature of the organism. Concentrations which have no effect upon one individual or a whole type will prevent growth and therefore reduction in another individual or another type. This explanation holds for members of the anaerobe group, the tuberculosis group, the diphtheria group, the colon-typhoid group and a few isolated individuals. In the cases of anaerobes, selenium dioxid or sodium selenite proves very inimical to growth. When, however, the medium contains very minute amounts of the selenium compound growth and reduction invariably result.

Proteus mirabilis and *B. phosphorescens*, according to Gosio's first communication, gave uncertain reactions. It was found that in repeating experiments with these two organisms, *P. mirabilis* grown on a sugar-free medium containing five or six drops of neutralized sodium selenite brought about good reduction within twenty-four hours. *B. phosphorescens* under similar conditions did not cause reduction.

The experiment with B. *phosphorescens* was repeated with smaller amounts of sodium selenite. Selenium dioxid was also tried as indicator. To tubes containing sugar-free medium was added 0.1 cc. of 0.2 per cent sodium selenite solution. Tubes were also made up with 0.1 cc. of 0.1 per cent selenium dioxid solution. At the end of three days reduction was noticeable in the phosphorescens culture treated with sodium selenite but not in the one containing the more toxic selenium dioxid. Evidently this organism responds very sensitively to selenium compounds, a fact which accounts for Gosio's negative finding. Growth was hardly noticeable.

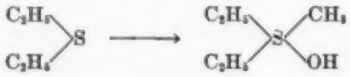
ALKYLATION BY MICROÖRGANISMS

We have seen that certain bacteria produce volatile compounds of characteristic odor from culture media containing

sodium selenite and that in some instances the brick red selenium formed by reduction is entirely removed by a biologic process of volatilization. This disappearance, which we believe is the result of alkylation, takes place very readily in cultures having extremely low concentrations of sodium selenite.

The researches of several investigators have established the fact that alkylation is not an unusual biologic process. His found that pyridine is converted in the biologic organism to methyl pyridyl ammonium hydroxid.

Neuberg and Grosser observed the formation of diethyl methyl sulfinium base from diethyl sulfid.



Pohl demonstrated the production from thiourea of diethyl sulfid.

Gosio was the first to show that certain molds grown upon media containing minute quantities of arsenic produce volatile compounds characterized by a garlic-like odor. Biginelli proved that the gas generated from arsenic cultures was completely absorbed by mercuric chlorid solution with the formation of colorless crystals of a double compound of diethyl arsine and mercuric chlorid, represented by the formula

$$As\,H(C_2H_5)_2\,2HgCl_2$$

Penicillium brevicaule, which Gosio isolated from the air, possessed this property in the highest degree. Indeed, this mold

may be regarded as a living reagent for arsenic. According to Abel and Buttenberg, quantities as minute as 0.000001 gram may be recognized with certainty. This test is so delicate that it has been found of great value in toxicological analysis in the preliminary examination for arsenic. It is considerably more delicate than Bettendorf's test and it may equal in sensitiveness the Marsh and the Gutzeit tests. The great advantage of the biological over the chemical method lies in the fact that less time is required to get results since the tedious destruction of organic matter is rendered unnecessary, and since, furthermore, a number of tests may be made at the same time.

The "arsenic molds" do not produce odoriferous volatile compounds from sulphur, phosphorus, boron, bismuth or antimony compounds, but they possess the power of converting selenium and tellurium compounds into volatile substances of characteristic odor. The odor from tellurium compounds resembles that arising from arsenic compounds, and is garlic-like, while the odor from selenium compounds is mercaptan-like. Selenium and tellurium may be differentiated from arsenic by the use of molds which do not alkylate the latter. According to Maassen 0.1 gram of sodium selenite or tellurite in 50 grams of culture medium inhibits or prevents the formation of the volatile compound, and 0.001 gram of sodium selenite or tellurite does not produce the odor. In order to get a positive test 50 grams of the nutrient medium should contain no less than 0.005 gram and not more than 0.1 gram of selenite or tellurite. The odor persists for months.

A number of workers—Japha, Rabuteau, Czapek and Weil, Hofmeister, Woodruff and Gies, Maassen, Jones, Filippi and Levine—have observed after the administration of selenium compounds to animals the presence of a volatile compound of characteristic odor emanating from the expired air. Gmelin, Hausen, Heeren, Rabuteau, Czapek and Weil, Hofmeister, Mead and Gies and others have observed a peculiar odor about their clothing, about the animals and in the room when experimenting with tellurium compounds. The odor was attributed by some (Rabuteau, Fillipi) to hydrogen selenid or tellurid while

others (Wöhler and his pupils) believed it to be due to ethyl selenid or tellurid. Hofmeister in an extensive research found methyl tellurid in the expired air of experimental animals. Reasoning by analogy, he concluded that the substance exhaled by animals dosed with compounds of selenium, was methyl selenid. Maassen corroborated the findings of Hofmeister in so far as higher animals were concerned. He proved, however, that while animals synthesized the methyl selenid or tellurid, microörganisms produced the ethyl selenid or tellurid.

MICROÖRGANISMS AS REAGENTS FOR SELENIUM COMPOUNDS

1. Alkylation through molds

There are two type methods in toxicological procedures for the detection of selenium. One type method depends upon purely chemical means. The tissue under investigation is oxidized, using as the oxidizing reagent any of the following: potassium hydroxid and potassium nitrate (Kletzinsky), aqua regia (Vohl), nitric acid (Quarelli, Levine), nitric and sulphuric acids (Duhamel and Juillard), potassium chlorate and hydrochloric acid (Levine). After removal of excess of the oxidant the resulting clear solution is boiled with hydrochloric acid to reduce any selenate to selenite.

$$Na_2SeO_4 + 2HCl \rightarrow Na_2SeO_3 + H_2O + Cl_2$$

The selenite is finally reduced to free selenium with sulphur dioxid (Kletzinsky, Quarelli, Vohl, Levine) or hydrazine hydrate (Duhamel and Juillard).

The other type method is biological. There are three distinct ways of detecting selenium biologically. One of these is through the use of molds. The detection of arsenic employing molds as biologic reagent has become a well established method as a result of the researches of Gosio, Abel and Buttenberg and others, and has now found its way into standard text books on toxicology. This mold method also works for selenium. It is necessary, however, to subject the tissues to oxidation before using the microörganic reagent. According to Rosenheim, free

selenium or tellurium, unlike free arsenic, is not alkylated by molds. Administration of selenium compounds to animals sets up according to Levine two processes of detoxication: one, removal of the poison by volatilization through a process of alkylation; the other, the reduction of the compound to inert, nontoxic, free selenium which deposits in the tissues. Before applying a mold like *Penicillium brevicaule*, the tissue is oxidized in order to convert any free selenium to the selenite form.

Quarelli was the first to make actual application of the mold method. He employed it in the analysis of tissues of a human being who had died of cancer, and who had received while still living, injections of colloidal selenium, a substance for which in cancer some clinicians claimed therapeutic value. The tissues were dried in a thermostat, powdered, suspended in water and treated with nitric acid. The selenium was thus converted to selenious acid. The excess of nitric acid was removed by evaporation. The mixture was finally neutralized with 0.1 N sodium hydroxid. Portions of the solution of tissue thus prepared were inoculated with mold, while other portions were acidified with hydrochloric acid and reduced to free, brick-red selenium by means of sulfur dioxid. The chemical and the biological method gave similar results. With both methods Quarelli obtained the greatest quantity of selenium in blood and liver and relatively smaller quantities in gall bladder, heart, aorta, stomach, pancreas and duodenum.

2. Reduction by bacteria

Scheuerlen, Klett, Gosio, Maassen and others have shown that sodium selenite is reduced by bacteria to brick-red selenium. This reduction we have also found to occur with selenious acid and with selenic acid, but not with its salts, the selenates. Potassium selenocyanid also yields free selenium, but this decomposition is not a vital phenomenon since acids in general and specifically acid metabolites in the culture medium bring about this result.

With sodium selenite the precipitated selenium can be very prettily observed in the medium along the path of growth.

Glucose accelerates the reducing process. One part of sodium selenite may be detected by reduction in 200,000 parts of culture medium. *B. coli* is especially suitable as a biologic reducer. The tissue containing selenium is digested with nitric acid. The excess thereof is removed by evaporation. The residue is boiled with hydrochloric acid to reduce any selenate to selenite. The resulting solution is evaporated to a small volume, neutralized and incorporated into glucose agar medium, which is finally inoculated with *B. coli*. The efficacy of the procedure was proven as follows: to tissues in 5 gram portions—muscle, liver, spleen and blood—were added 0.0005, 0.001 and 0.002 gram of sodium selenite. These were treated in the manner described and the final residues mixed with 10 grams of culture medium. Inoculated with *B. coli* the media showed a streak of red selenium following the line of growth in the stab culture.

Experiments *in vivo* were also performed. One rabbit was given subcutaneously 0.0005 gram, while another was given 0.001 gram of sodium selenite per kilo. Portions of the liver of these animals were oxidized. Selenium was detected by the formation of brick-red selenium through reduction with sulphur dioxid of one-half of the oxidized tissue residue acidified with hydrochloric acid. The other half was incorporated in culture media, which were inoculated with *B. coli*. The brick-red line following the path of growth indicated the presence of selenium. Portions of the liver were put in 5 per cent formaldehyde, sectioned and stained with eosin and hematoxylin. The liver cells had degenerated to such an extent that they stained very poorly. Chocolate red granules of selenium were found intracellularly and extracellulary.

The histological method, which constitutes a third biological method, was used by Jones to demonstrate the reduction of selenium compounds in the animal organism. Levine has elaborated this method as a toxicological procedure. Dogs and rabbits given sublethal and lethal doses of selenium compounds show reduction in some tissues, especially in the liver. He recommends the histological examination of the liver and other tissues as a routine procedure in the toxicological analysis of tissue for selenium.

THE NATURE OF BACTERIAL REDUCTION

Reduction is brought about by the bacterial cell. This fact was proven by Spina and Rothberger. According to these investigators, heating cultures at 60° to 70°C. destroys the reducing power. Gosio found no reduction in dead cultures. Smith did not observe reduction in filtered culture although Hahn and Cathcart, working with methylene blue, found filtrates to be reducing in action. According to Cahen, Spina, Smith, Rothberger, Klett, Beijerinck and Maassen, reduction is due to the direct activity of the bacterial cell. Roszahegyi, Baginsky, Müller, Wolff, and Wichern conclude, however, that reduction results not directly from the bacterial protoplasm but from its products of metabolism. The later findings, those of Kanaido, indicate that reduction is a vital process and is inhibited by rabbit's normal serum and to a very marked degree by homologous immune sera.

The reducing power of bacteria has been ascribed by recent investigators to an enzyme named reductase. Sterile bacterial extracts are known to have reducing power. Avery and Neill obtained reduction of methylene blue from sterile broth extracts of unwashed pneumococci entirely free from living or intact cells. There has been a great deal of discussion regarding the nature of bacterial reductase. The question whether it is an exo-enzyme or an endo-enzyme or whether such a thing as bacterial reductase exists, has been answered both affirmatively and negatively. Experiments undertaken by Kligler and Levine with the intention of determining these points, indicated that reduction was closely associated with the life of the cell, and that the more vigorous the growth the more marked was the reduction. The filtrate of a broth culture of *B. coli* passed through a Berkefeld failed to reduce selenium dioxid. In answer to the objection that the Berkefeld withheld certain substances, such as a co-activator or the enzyme possessing reduction properties, we point to the lack of reduction in the agar tube outside of the zone of growth. If there were such a substance as a soluble bacterial reductase or some metabolic by-product of reducing nature, its gradual diffusion throughout the

medium should cause diffuse reduction. Furthermore, a killed broth culture of *B. coli* added to the solution of selenite failed to show any reduction even after two weeks. Finally a large mass growth of an actively reducing strain of *B. coli* was dried in a desiccator triturated with sand, taken up with physiological salt solution and centrifuged. The supernatant liquid (1) kept under toluol to prevent growth, together with a solution of sodium selenite to make up a concentration of 1:10,000, failed to bring about any reduction. The residue (2) was extracted with a mixture of glycerol and water. This extract (3) had no reducing action on sodium selenite. The mass (4) remaining after the glycerol extraction was also brought into contact with sodium selenite but no reducing action was apparent. However, a combination of supernatant liquid (1) and residue (2) showed decided ability to reduce.

Harden and Zilva believe that the reducing enzyme is not a single principle but a system consisting of enzyme plus acceptor or activator. They found that by washing with saline *Bacillus coli-communis* loses its activator or acceptor and is no longer able to reduce methylene blue. Addition of the boiled washings restores, however, the lost function. Avery and Neill found that washing pneumococci in phosphate solution also renders them incapable of reducing methylene blue. Upon the addition of meat infusion or extract these washed bacterial cells recovered their reducing power. To determine the nature of the activator or acceptor, Harden and Zilva added a great variety of substances to their washed organisms. They obtained positive results with a great number of the substances used. The following list gives the results with the compounds and mixtures tested:

Positive

Boiled washings	Mannitol
Bouillon	Alanine
Horse serum	Asparagine
Liebig's extract	Albumin
Arabinose	Globulin
Xylose	Peptone (Witte)
Glucose	Sodium acetate

Levulose	Sodium formate
Galactose	Sodium succinate
Mannose	Sodium lactate
Maltose	Sodium pyruvate
Inulin	Sodium glycerate
Glycerol	Isobutyric acid

Negative

Formaldehyd	Potassium oxalate
Acetaldehyd	Rochelle Salts
Propylaldehyd	Sodium citrate
Isovaleraldehyd	Hydroxybutyric acid
Cinnamylaldehyd	Linolinic acid
Salicylaldehyd	Butyl alcohol
Lactose	Phloroglucinol
Raffinose	Creatin
Erythrol	Guanidin hydrochlorid
Dulcitol	Hypoxanthin
Sorbitol	Xanthin
Oenanthol	Adenin
Glycocol	

Bach maintains that simple aldehydes possess the power to activate. According to him, amino acids may also function, since these are readily converted to aldehyd. Harden and Zilva's experiments, however, proved negative with aldehydes. Bach claims that complex aldehydes like carbohydrates do not activate. Harden and Zilva found a number of carbohydrates capable of restoring reducing power.

Abelous and Aloy found that many substances besides aldehydes act as activators or co-ferments. Among others may be mentioned benzylamin, dibenzylamin, substances with heterocyclic groups, such as quinolin, the terpene hydrocarbons and such inorganic compounds as manganese salts. Harden and Norris found with washed yeast a number of substances capable of playing the rôle of activator or acceptor. Such were benzaldehyd, salicylaldehyd, anisicaldehyd, isovaleraldehyd, dihydroxyacetone, succinic acid, glycollic acid, lactic acid, sodium lactate, citric acid, glyceric acid, bouillon, boiled yeast extract, normal horse serum and sterile milk. Negative results were obtained with glycol, propylene glycol, 2-3 butylene glycol, glycerol, pyrogallol, resorcinol, quinol, acetone, formaldehyd, acetaldehyd, methylglyoxal, citral, glucose, levulose, pyruvic

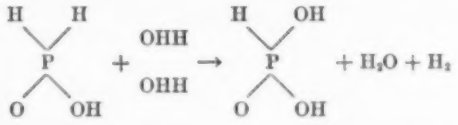
acid, formic acid, acetic acid, malic acid, tartaric acid, mandelic acid, yeast nucleic acid, creatine, guanidin, p-phenylenediamin, glycocol, alanine, aspargine, tyrosine, and Witte's peptone.

The number of compounds that have the power to re-activate the reducing enzyme present such a variety of chemical types that it is difficult to determine a specific chemical nature for the compounds functioning as activators. It is very likely that the rôle of acceptor or activator may be played by any compound that has the power to take up with ease the oxygen removed from the substrate or zymolyte undergoing reduction.

There seem to be some discrepancies in the results of the investigators cited. Harden and Norris found that acetaldehyd activates washed muscle but not washed yeast. Harden and Zilva found that benzaldehyd, anisealdehyd and salicylaldehyd activate washed yeast, although Harden and Norris report that these same compounds show a negative behavior towards washed *Bacillus coli*. Glycerol, glucose, levulose, alanine, asparagine and peptone were negative with yeast but positive with *Bacillus coli*. The experiments of Harden and his collaborators were performed qualitatively, without regard to concentration of added substance or of hydrogen ions. It may be possible that by regulating these factors, the results of Bach and of Harden and his associates would attain some semblance of agreement. The point in reference to concentration may be illustrated from the very experiments of Harden and Zilva. They found that the addition of increasing quantities of broth or of glycerol increased the rate of reduction until a certain optimum, after which any further increase in the quantity of broth or glycerol diminished the rate of reduction.

Bach attributes the reducing action of plant or animal organism to a system in which enzyme, substrate, acceptor and water in a state of dissociation each plays a specific rôle. The dissociating process furnishes the hydrogen to reduce the methylene blue or nitrate as well as the oxygen to oxidize the acceptor (aldehyd). In other words, biologic reduction is a complex phenomenon, a hydrolytic-oxidative-reduction process, of which the Cannizzaro reaction is an example.

Bach's explanation becomes more plausible if we consider an analogous case—the oxidation of hyposulfites, which he himself investigated. In water alone hyposulfites do not undergo oxidation, at least at any measurable rate. But in the presence of a catalyst (finely divided palladium) this does take place. The water is decomposed and its HO used for oxidation of the hyposulfite. The hydrogen is taken up temporarily by the palladium and then set free. An easily reducible substance, if present, will be reduced by the nascent hydrogen.



When aldehydes are taken instead of hyposulfite, it is observed that the metals of the platinum group do not to any appreciable extent accelerate the decomposition of water and hence the oxidation of the aldehyd. It is only after the addition of an easily reducible substance, such as methylene blue, indigo or nitrate, which acts as an acceptor for nascent hydrogen, that acceleration occurs. Thus, formaldehyd in the presence of a catalytic agent and acceptor like methylene blue is of some theoretical interest. Since this dye contains no oxygen it follows that the atom of this element necessary to convert formaldehyd into formic acid must come from water, the only source of oxygen in the reaction system.

The reducing enzyme bears an interesting analogy to the oxidizing enzyme. If we regard the oxidizing system as

peroxidase + peroxide

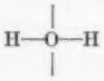
we may look upon the reducing system as

Schardinger enzyme + aldehyd

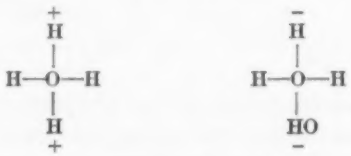
An oxidase produces its oxidation by the aid of gaseous oxygen, while a reductase acts through the intermediation of the combined oxygen in water.

Bach's explanation of the part taken by water in the reducing

phenomenon lies in the consideration of water as an unsaturated compound in which oxygen is tetravalent.



Since ions are believed to be associated with water molecules to form unstable complexes, and since hydrogen and hydrogen ions are present in water, it is probable that two compounds are formed, one, water in combination with hydrogen ions, the other, water in combination with hydroxyl ions.



The first may be named hydrogen suboxide or oxygen perhydride. It bears analogy to the metallic salts of the type M_4O, an example of which is Ag_4O. The second is the hydrate of hydrogen peroxide ($H_2O\ (OH')_2$).

The acceleration of the oxidation of aldehydes in the presence of platinum, Engler and Wöhler explain on the ground that the colloidal metal unites with oxygen to form a peroxide, PtO_2, which reacts with water to form the hydrate, HO – Pt – O –O, which acts as a powerful oxidizing agent. This compound is also supposed to be produced by the interaction of platinum and the $H_2O\ (OH')_2$ present in water. Since the latter is present in a small amount, it is quickly used up, the equilibrium is disturbed, and more is therefore formed, with the result that the catalytic process continues. It is not unlikely that the platinum metals would form strongly reducing hydrides by interaction with oxygen perhydride (H_4O) if its presence in water is admitted.

Bach believes that the reducing enzyme or reductase activates oxygen perhydride just as peroxidase causes the activation of peroxide oxygen. For this reason he calls the reducing enzyme,

perhydridase. Bach's view, although it explains the occurrence of active hydrogen, is built on a weak foundation for it supposes the existence of compounds of doubtful nature.

Differing somewhat from Bach's conception of biologic reduction is that formulated by Wieland. This investigator sought the essential cause of certain typical biologic oxidations in the activation of oxygen, not in the activation of hydrogen. According to his conclusions oxidases, reductases and mutases have no separate existence.

His views may be illustrated from his conclusions derived from a quantitative study of the action of Schardinger's enzyme on salicylaldehyd. The aldehyd is assumed to act in the form of its hydrate and the enzyme activates two of its hydrogen atoms which are thus rendered available for an acceptor. This acceptor may be (1) molecular oxygen, (2) an easily reducible substance like methylene blue, or (3) a molecule of the aldehyd in the unhydrated form. If molecular oxygen is the acceptor, the result is the oxidation of the aldehyd. If an easily reducible substance is the acceptor, its reduction is associated with indirect oxidation of the aldehyd. If unhydrated salicylaldehyd is the acceptor, the Cannizzaro reaction, the mutase effect, is observed.

$$R\,CH\begin{matrix}OH\\OH\end{matrix} + R\,CHO \longrightarrow R\,COOH + R\,CH_2OH$$

Whether any one of these results is obtained or a mixture of all three, depends upon the concentration and speed of reaction of the various acceptors. The most important hydrogen acceptor is oxygen but other acceptors may be equally important for other aspects of cell activity. Thunberg, from a series of experiments, has been led to adopt Wieland's conception. According to the latter methylene blue acts as a hydrogen acceptor. The hydrogen comes from a substance in the tissue, which acts as a hydrogen donator and the enzyme itself plays the rôle of a hydrogen transportase.

Some investigators hold that reduction through the intervention of enzymes is not the only mechanism for biologic re-

duction. Heffter is of the belief that a great part of the phenomenon relating to the oxidizing and reducing properties of protoplasm is inherent in the sulfhydryl group. Mathews and Walker have demonstrated the ease with which cystein, $CH_2(SH)$ $CH(NH_2)$ COOH undergoes oxidation. Arnold has shown that tissues give the sodium nitroprussid reaction indicating the presence of the sulfhydryl grouping (SH). He has also shown that a number of proteins give this reaction and that in the absence of protein, a positive response is elicited from cystein.

Very recently, F. G. Hopkins has isolated from yeast, muscle and liver a compound giving the nitroprussid reaction. It is a dipeptide of glutamic acid and cystein. It has reducing properties similar to those ascribed by de Rey-Pailhade to his philothin of yeast. Hopkins by way of analogy calls his dipeptide glutathione. The sulfhydryl group (SH) of its cystein fraction readily gives up its labile hydrogen. It, therefore, acts as an oxygen acceptor or hydrogen donator. The dipeptide, when in the oxidized or disulfid form (S-S) may act as a hydrogen acceptor.

$$2RSH + O_2 \longrightarrow R{-}S{-}S{-}R + H_2O_2$$

$$2RSH + H_2O_2 \longrightarrow R{-}S{-}S{-}R + 2H_2O$$

$$R{-}S{-}S{-}R + H_2 \longrightarrow 2RSH$$

It is in this play of oxidizing and reducing effects of cystein and cystine that may be found, at least in part, the explanation for the affinity of the cells for oxygen and the possibility of the formation of peroxid. Avery and Neill in their work on the pneumococcus suggest that peroxid formation and methylene blue reduction are functions of the same or closely related systems, though the particular action induced depends upon whether molecular oxygen or methylene blue serves as a hydrogen acceptor or oxygen donator.

Nascent hydrogen itself may account for some biologic reductions. Hoppe-Seyler has demonstrated by bacterial intervention the formation of carbon dioxid and the very potent reducing agent, hydrogen, from formic acid, glycolic acid, lactic and glyceric acids. He found that those compounds which did

not evolve free hydrogen, like malic acid, tartaric acid and asparagin, were reduced in part to succinic acid. A number of workers have proven the reduction by anaerobes of amino acids with the formation of saturated fatty acid and ammonia. This calls into interaction two atoms of hydrogen.

$$R\,CHNH_2\,COOH + H_2 \longrightarrow R\,CH_3\,COOH + NH_3$$

Very recently, Neuberg and Neuberg and Rewald have shown that among the products of bacterial decomposition of α-ketonic acids may be found formic acid, carbon dioxid and hydrogen. Carbohydrates may also yield hydrogen since they have the power to produce as a result of biologic fragmentation, the very acids that evolve this reducing gas.

Certain tissue compounds also possess reducing power. Hasse has shown that amino acids, particularly glycine, have the power to reduce methylene blue. Of special interest are the investigations of Fränkel and his associates in relation to the strong reducing action of tissue phosphatids. Compounds associated with intermediate metabolism—formic acid, lactic acid, aldehydes and others—are known for their strong reducing action. Formaldehyd, in minute quantities, is found in plant tissue and acetaldehyd has been proven by Neuberg and Nord to be an intermediate product in the bacterial fermentation of glucose, mannitol or glycerol.

Several investigators have shown that in the presence of carbohydrates bacteria show greater reducing capacity. This accelerated effect may be due to the carbohydrate itself or to its fragmentation products. The reducing action at 37.5°C. of carbohydrates with a free carbonyl group in the absence of tissue has been demonstrated by Levine by the use of sodium selenite in alkaline medium and even by the use of the ordinary Fehling and Fehling-Benedict reagents. Liver pulp, boiled and unboiled, also reduced sodium selenite. Compounds closely associated with carbohydrates as products of their decomposition—aldehydes, ketones, formic and lactic acids—reduced sodium selenite in the presence or absence of tissue but in a slightly acid medium.

Indeed, it may be stated that most of the organic compounds apt to be present in biologic material show greater or less reducing tendencies, although these are not manifested with the ordinary reagents nor at biologic temperatures. Levine with the use of sodium orthovanadate and Levine and Jahr with the use of ammonium molybdate have shown that almost all types of organic compounds of biologic significance—unsaturated fatty acids, glycerol, lipins, amino acids, proteins, carbohydrates, aldehydes, ketones, phenols, uric acid and creatinin—are capable of reducing. Plant and animal tissues heated or unheated, kept at 37.5°C. easily reduce ammonium molybdate. The reduction of potassium permanganate is not necessarily enzymatic in character. Levine has shown that boiled or unboiled diffusate from yeast suspension, bacterial cultures or liver, as well as the filtrate obtained by alcoholic precipitation, bring about at ordinary temperature the instantaneous reduction of permanganate. Yet in spite of all that may be said in favor of the reducing action of the various chemical components of tissue, we must not overlook the presence or underestimate the importance of enzymes responsible for reduction.

The author gratefully acknowledges his indebtedness to Professor William J. Gies for his valuable suggestions and helpful criticisms and to Dr. I. J. Kligler and Mr. William Rothberg for their valuable coöperation.

SUMMARY

Living bacteria bring about the reduction of selenious acid (Chabrié and Lapicque, Levine), selenic acid (Levine) and sodium selenite (Scheuerlen, Klett, Gosio, Levine). Sodium selenate is not reduced (Klett, Levine); nor is potassium selenocyanid (Levine). Due to decomposition induced by acids, metabolically produced, selenium may, however, be deposited from potassium selenocyanid, as in the case of *B. coli* cultures (Levine). Heated cultures do not reduce (Klett, Gosio, Levine). The media employed should not contain chemical reducing substances, such as glucose or lactose (Gosio, Gloger, Levine).

Reduction is a vital process (Cahen, Spina, Smith, Roth-

berger, Klett, Maassen, Gosio, Levine); it is an intracellular process (Kligler, Levine, Harden, Zilva, Kanaido). The reductase elaborated by the bacterial cell is an endo-enzyme reducing energetically in the presence of an activating substance or co-enzyme, which is capable of being dissolved out from the cell or of being removed by Berkefeld filtration (Kligler and Levine, Harden and Zilva).

Selenious acid, sodium selenite and selenic acid retard growth. The extent of retardation depends upon the concentration and chemical nature of the selenium compound and upon the individuality of the organism. *Streptococcus pyogenes* is more sensitive than *B. coli*. The anaerobes of symptomatic anthrax, oedema and tetanus are extremely sensitive and growth does not take place except in minute concentrations of the above selenium compounds (Levine).

Sodium selenate and potassium selenocyanid in the quantities used show but slight retarding effect on growth (Levine).

Reduction is directly proportional to the intensity of growth (Klett, Gosio, Levine). When there is no growth there is no reduction (Klett, Gosio, Levine), but when the concentration of selenium compound is very small (1:200,000) there may be growth without visible evidence of reduction (Levine). This lack of visible selenium may be due to its removal by volatilization. With higher concentration of selenium compounds in the culture medium the activity of reduction outbalances that of alkylation (Levine).

Selenium dioxid or sodium selenite cannot be used as a differential test between aerobes and anaerobes, since both types reduce (Levine).

There is no specific relation between reduction and formation of hydrogen sulfid, as Gloger maintained, since organisms such as *B. acidilactici*, *B. pseudodiphtheriae* or *B. tuberculosis*, that produce no hydrogen sulfid or only faint traces, are capable of reducing selenium dioxid or sodium selenite (Levine).

The diphtheria organisms have been tested with different concentrations of selenium dioxid and have been found to be efficient reducers. In the very high concentrations some or-

ganisms failed to grow and therefore gave no evidence of reduction (Levine).

The reducing action on sodium selenite in very high concentration (1:10,000) by the various organisms in the colon typhoid group may be of practical value in differentiating one type from another. *B. paratyphi* B reduces while *B. paratyphi* A does not (Levine). This difference in action harmonizes with the findings of Burnet and Weissenbach, Jordan and Victorson and also Kligler. These investigators distinguished these two types of organisms by the use of lead acetate media, which made apparent the difference in the reducing action as manifested by the production of hydrogen sulfid.

Microörganisms can be used as living reagents in the toxicological analysis for selenium. With the aid of certain alkylating molds, selenium can be detected by means of the characteristic and persistent odor of ethyl selenid (Quarelli). With the aid of bacteria that possess intense reducing activity, selenium compounds, in the form of selenite ion, can be identified by the brick-red line or streak following the path of growth in a stab culture (Levine).

Selenium compounds serve as better indicators for reducing enzymes than organic dyes. Since the reduction is localized in the bacterial zone of growth, it cannot be ascribed to metabolic products. Unlike the reduction of dyes, the decomposition of selenium compounds to free selenium is an irreversible reaction and the precipitated element shows no tendency to re-oxidize (Levine).

For practical purposes selenium dioxid or sodium selenite in a concentration of 1:50,000 or 1:25,000 can be used to demonstrate bacterial reduction in a solid sugar-free culture medium (Levine).

Selenium agar (0.15 per cent Na_2SeO_3) as a culture medium for the selective growth of typhoid bacilli is superior to malachite green or Endo agar according to Guth.

REFERENCES

ABEL, R., AND BUTTENBERG, J. 1899 Ueber die Einwirkung von Schimmelpilzen auf Arsen und seine Verbindungen. Zeitschr. f. Hyg. u. Infektionskrankh., **32**, 449.

ABELOUS AND ALOY 1917 Sur les phénomenès biochimique d'oxydo-réduction. Comptes rendus des séances de l'académie des sciences, **165**, 270.

ACKERMANN, D. 1908 Ein Faülnisversuch mit Arginin. Zeitschr. f. physiol. Chemie, **56**, 305.

ACKERMANN, D. 1909 Ueber die Entstehung von Faülnisbasen. Zeitschr. f. physiol. Chemie, **60**, 482.

ACKERMANN, D. 1910 Ueber den bakteriellen Abbau des Histidins. Zeitschr. f. physiol. Chemie, **65**, 504.

ACKERMAN, D. 1911 Die Sprèngung des Pyrrolidinringes durch Bakterien. Zeitschr. f. Biol., **57**, 104.

ACKERMAN, D., AND KUTSCHER, F. 1910 Ueber die Aporrhegmen. Zeitschr. f. physiol. Chemie, **69**, 273.

ALSBERG, C. L. 1915 Note on the reduction of oxyhemocyanin in the serum of *Limulus polyhemus* L. Jour. Biol. Chem., **23**, 495.

ARNOLD, V. 1911 Eine Farbenreaktion von Eiweisskörpern mit Nitroprussidnatrium. Zeitschr. f. physiol. Chemie, **70**, 300.

ARNOLD, V. 1911 Ueber den Cysteingehalt der thierischen Organe. Zeitschr. f. physiol. Chemie, **70**, 314.

AVERY, O. T., AND NEILL, J. W. 1924 Studies on oxidation and reduction by pneumococcus. III. Reduction of methylene blue by sterile extracts of pneumococcus. Jour. Exper. Med. **39**, 543.

BACH, A. 1911 Zur Kenntnis der Reduktionsfermente. I Mitteilung. Ueber das Schardinger Enzym (Perhydridase). Biochem. Zeitschr., **31**, 443.

BACH, A. 1911 Zur Kenntnis der Reduktionsfermente. II Mitteilung. Reduktion der Nitrate durch das System Perhydridase-Aldehyd-Wasser. Biochem. Zeitschr., **33**, 282.

BACH, A. 1912 Zur Kenntnis der Reduktionsfermente. III Mitteilung. Vorkommen eines Kofermentes in tierischen Geweben. Biochem. Zeitschr., **38**, 154.

BACH, A. 1913 Zur Kenntnis der Reduktionsfermente. IV Mitteilung. Pflanzliche Perhydridase. Biochem. Zeitschr. **52**, 412.

BACH, A. 1914 Zur Kenntnis der Reduktionsfermente. V Mitteilung. Bildung von Aldehyden aus Aminosaüren. Biochemi. Zeitschr. **58**, 205.

BAGINSKY, A. 1888 Ueber Gährunsvorgänge im kindlichen Darmcanal. Deutsche medizin. Wochenschr. **14**, 391.

BARR AND BUCHANAN. 1912 Iowa Stage College Engineering Experiment Station, Bulletin 26.

BEHRING. 1889 Beiträge zur Aetiologie des Milzbrandes. VII. Ueber Lackmusgefärbte Agarnährboden. Zeitschr. f. Hyg. u. Infektionskrankh. **7**, 177.

BELFANTI, S. 1912 Die vitale Reakton nach Gosio beim Tuberkelbazillus. Zeitschr. f. Chemotherapie u. verwande Gebiete **1**, 113.

BRASCH, W. 1909 Ueber den bakteriellen Abbau primärer Eiweissspaltungsprodukte. Biochem. Zeitschr. **18**, 380.

Brasch, W. 1909 Weitere Untersuchungen über den bakteriellen Abbau primärer Eiweissspaltungsprodukte. Biochemi. Zeitschr. **22,** 403.

Brenner, W. 1916 Züchtungsversuche einiger in Schlamm lebenden Bakterien Auf Selenhaltigen Nahrboden. Jahrb. f. wissenschaftliche Botanik, 19, **57,** 95.

Cahen, F. 1887 Ueber das Reduktionsvermögen der Bakerien. Zeitschr. f. Hyg. u. Infektionskrankh. **2,** 386.

Capaldi, A., and Proskauer, B. 1896 Beiträge zur Kenntnis der Säurebildung bei Typhusbacillen und *Bacterium coli*. Zeitschr. f. Hyg. u. Infektionskrankh., **23,** 452.

Cathcart, E., and Hahn, M. 1902 Ueber die reduzirenden Wirkungen der Bakterien. Arch. f. Hyg. **44,** 295.

Chabrié, C., and Lapicque, L. 1890 Sur l'action physiologique de l'acide sélénieux. Compt. rend. des séances de l'académie des sciences, **110,** 152.

Conradi, H. 1913 Ueber ein neues Prinzip der elektiven Züchtung und seine Anwendung bei Diptherie. Centralblatt für Bakteriologie, II Abtheilung, Referate **57,** 93.

Czapek, F., and Weil, J. 1893 Ueber die Wirkung des Selens und Tellurs auf den thierischen Organismus. Arch. f. exper. Pathol. u. Pharmokol., **32,** 438.

Dakin, H. D. 1922 Oxidations and Reductions in the Animal Body, p. 98.

van Delden, A. 1903 Beitrage zur Kenntnis der Sulfatreduktion durch Bakterien. Centralbl. f. Bakteriol., 11 Abt., **11,** 81.

Dieudonné. 1894 Zusammenfassende Uebersicht über die in den letzer zwei Jahren gefunden cholerasähnlichen Vibrionen. Centralbl. f. Bakteriol., Abt. 1, **16,** 363.

Duhamel, B. G., and Juillard, M. 1912 Localization du sélénium colloidal électriques dans les organes. Comptes rendus de la société de biologie, **72,** 714.

Engler, C., and Wöhler, L. 1901 Pseudokatalytische Sauerstoffübertragung. Zeitschr. f. anorganische Chemie, **29,** 1.

Elsner 1894 Untersuchungen uber electives Wachsthum der Bacterien coliarten und des cholerasähnlichen Vibrionen. Centralbl. f. Bakteriol. Abt. 1. **16,** 363.

Filippi, E. 1913 Recherche farmocologicle sopra il selenio e i suoi composte Lo Sperimentale, Firenze, **67,** 565.

Gasser, J. 1890 Culture du bacille typhique sur milieux nutfitifs colorés. Arch. du médecin experimentale et d'anatome pathologique **2,** 750.

Gayon, U., and Dubourge, E. 1894 Sur les vins mannités. Annales de l'institut Pasteur, **8,** 108.

Gayon, U., and Dubourge, E. 1901 Nouvelles recherches sur le ferment mannitique. Annales de l'institut Pasteur, **15,** 525.

Gies, W. 1902 Chemical changes in the body in which the methyl group may be involved. Therapeutic Monthly, **2,** 144.

Gloger, R. 1906 Kallium tellurosum in der Medizin und Hygiene. Centralbl. f. Bakteriol. **40,** 584.

Gmelin, Chi. 1824 Versuche über die Wirkungen des Baryts, Strontians u.s.w. auf den thierischen Organismus, Tubingen, p. 43.

GOPPELSRÖDER, F. 1862 Beitrage zum Studium der Salpeterbildungen. Poggendorf's Annalen, **115**, 125.

GOSIO, B. 1904 Sulla decomposizione di sali del telurio per opera dei microorganismi. Rendice Revista Accadamia dei Lincei. Seduita del 24 aprile; seduita del 4 guigno.

GOSIO, B. 1905 I telluriti e seleniti come rivelatori d'inquinamenti batterici. Rendice Revista Accademia dei Lincei. Seduita del 6 agosto.

GOSIO, B. 1905 Indikatoren des Bakterien Lebens und ihre praktische Bedeutung. Zeitschr. f. Hyg. u. Infektionskrankh. **51**, 65.

GOSIO, B. 1892 Azione di alcune muffe sui composti fissi d'arsenico. Rivista d'igiene, e sanita publica **3**, 201.

GUTH, F. 1916 Selennährboden für die elektive Züchtung von Typhus-bacillen. Centralbl. f. Bakteriol. 1 Abt., **77**, 487.

HAENDEL, AND THEODORASCU. Unpublished findings cited by Guth.

HAGGARD, H. W. 1921 The fate of sulfides in the blood. Jour. Biol. Chem., **49**, 519.

HARDEN, A., AND NORRIS, R. V. 1914 The enzymes of washed zymin and dried yeast (Lebedoff). II. Reductase. Biochem. Jour. **8**, 100.

HARDEN, A., AND ZILVA, S. S. 1915 The reducing enzyme of *Bacillus coli communis*. Biochem. Jour. **9**, 379.

HASSE, F. 1919 Ueber die Methylenblaureduktion durch Glycin. Biochem. Zeitschr. **98**, 159.

HAUSEN, K. 1853 Versuche über die Wirkung des Tellurs auf den lebenden Organismus. Annalen der Chemie und Pharmacie, **86**, 208.

HEEREN, M. 1861 Ueber Telluräthyl und Tellurmethylverbindungen. Chem. Centralbl., **6**, 916.

HEFFTER, A. 1907 Die reduzierenden Bestandteile der Zellen. Medizinisches naturwissenschaftliche Archiv., **1**, 82.

HELMOHOLTZ. 1843 Ueber das Wesen der Fäulnis und Gährung. Archiv. f. Anatomie, Physiologie und wissenschaftlichte Medicin, p. 453.

HIS, W. 1887 Ueber das Stoffwechselsproduct des Pyridins. Arch. f. exper. Pathol. u. Pharmakol., **22**, 253.

HOFMEISTER, F. 1904 Ueber Methyliering im Thierkörper. Archiv für exper. Pathol. u. Pharmakol. **12**, 457.

HOPKINS, F. G. 1921 On an auto-oxidisable constituent of the cell. Biochem. Jour. **15**, 286.

HOPPE-SEYLER, F. 1878 Ueber Gärungsprozesse. Zeitschr. f. physiol. Chemie, **79**, 2, 1.

JAPHA, A. 1842 Experiementa nonulla de vi selenii in organismum animalem. Dissertation, Halle.

JOACHIMOGLU, G. 1920 Zur Pharmakologie des Selens und Tellurs. I. Mitteilung. Die Wirkung ihrer Säuren auf Bakterien. Biochem. Zeitschr., **107**, 300.

JOACHIMOGLU, G., AND HIROSE, W. 1921 Zur Pharmakologie des Selens und Tellurs II. Mitteilung. Wirkung ihrer Säuren auf Diptheriebacillen. Biochem. Zeitschr., **125**, 1.

JONES, C. O. 1909 The physiological effects of selenium compounds with relation to their action on glycogen and sugar derivatives. Brit. Biochem. Jour. **4**, 405.

JORDAN, E. O., AND VICTORSON, R. 1917 Differentiation of the paratyphoid enteritides group. Jour. Infect. Dis. **21**, 554.

KANAIDO, S. 1916 Reduction of methylene blue by bacteria inhibited by immune sera. Saikiu Gaku (Zasshi, No. 243, 169; Japanese Medical Literature 2 (Part 1), 2–3 (1917).

KING, W. E., AND DAVIS, L. 1914 Potassium tellurite as an indicator of microbial life. Amer. Jour. Pub. Health **4**, 917.

KITASATO, S., AND WEYL, Th. 1890 Zur Kenntnis der Anaeroben. Zeitschr. f. Hyg. u. Infektionskrankh., **8**, 41; **9**, 97.

KLETT, A. 1900 Zur Kenntnis der reduzirenden Eigenschaften der Bakterien. Zeitschr. f. Hyg. u. Infektionskrankh. **33**, 137.

KLETZINSKY, V. 1858 Ueber die Ausscheidung der Metalle in den Secreten. Wiener medizin. Wochenschr. **1**, 356.

KLIGLER, I. J. 1917 Simple medium for the rapid differentiation of typhoid and paratyphoid bacilli. Amer. Jour. Pub. Health **7**, 805, 1042.

KLIGLER, I. J. 1913 The source of ammonia and nitrate in nitrate-peptone solutions, Amer. Jour. Pub. Health, **3**, 788.

KLIGLER, I. J., AND LEVINE, V. E. 1915 The Scheurelen-Klett selenium reaction in the diphtheria group. Biochem. Bull., **4**, 196.

KLIGLER, I. J., AND LEVINE, V. E. 1915 The nature of bacterial reductase. Unpublished communication.

KLIGLER, I. J., GREENBERG, D., AND LEVINE, V. E. Experiments with colon-typhoid bacilli grown on selenium medium. Unpublished communication.

KOCHMANN, R. 1920 Uber Schwefelwasserstoffbildung aus Sulfaten durch Faeces. Biochem. Zeitschr. **112**, 1255.

KRUSE, W. 1910 Allegemeine Mikrobiologie, Leipzig, p. 659.

LABBÉ, M. 1903 Action comparée des microbes at des toxines microbiennes sur le sang défibriné. Compt. rend. de la société de biologie **55**, 201.

LAURENT, E. 1890 Expérience sur la réduction des nitrates par les végétaux. Annals de l'institut Pasteur, **4**, 722.

LEVINE, V. E. 1913 Biochemical studies of selenium. Biochem. Bull., **2**, 552.

LEVINE, V. E. 1914 Ph. D. Dissertation, Columbia University.

LEVINE, V. E. 1914 Reduction of selenium compounds in the living organism. Biochem. Bull. **3**, 463.

LEVINE, V. E. 1915 Ann. New York Acad. Sci., **26**, 385.

LEVINE, V. E. 1915 Sodium selenite as a laboratory reagent for reducing substances. Biochem. Bull., **4**, 217.

LEVINE, V. E. 1915 The reducing power of anaerobes. Biochem. Bull., **4**, 215.

LEVINE, V. E. Reduction of permanganate by biologic tissue. Unpublished communication.

LEVINE, V. E. Reductions with compounds of the rarer elements II Sodium ortho-vanadate. Unpublished communication.

LEVINE, V. E., AND AHANA, W. W. Potassium selenocyanid as an indicator for free acidity. Unpublished communication.

LEVINE, V. E., AND JAHR, H. M. 1915 Reductions with compounds of the rarer elements. I Ammonium molybdate. Biochem. Bull., **4**, 224.

LEVINE, V. E., AND JAHR, H. M. 1921 The reducing action of micro-organisms on ammonium molybdate. Abstr. Bacteriol., **5**, 4.

LIPMAN, J. G., AND WAKSMAN, S. A. 1923 The oxidation of selenium by a new group of autotrophic microörganisms. Science, **58**, 60.

LUNKEWICZ, M. 1894 Eine Farbenreaktion auf die salpetrige Säure der Kulturen der Cholerbacillen und einiger anderen Bakterien. Centralbl. f. Bakteriol., Abt. 1, **16**, 9, 945.

MAASSEN, A. 1901 Die Zersetzung der Nitrate und der Nitrite durch die Bakterien. Arbeit. aus dem kaiserlichen Gesundheitsamte, **18**, 1.

MAASEN, A. 1901 Die biologische Methode Gosios zum Nachweis des Arsens und die Bildung organischer Arsen-Selen-und Tellurverbindungen. Arbeit. aus dem Kaiserlichen Gesundheitsamte, **18**, 1.

MAASSEN, A. 1904 Uber das Reduktionsvermogen der Bakterien und uber reduzirende Stoffe in pflanzlichen und tierischen Zellen. Arbeit. aus dem kaiserlichen Gesundheitsamte, **21**, 377.

MARPMANN. 1894 Zur Unterscheidung des *Bacillus typhi abdominalis* vom *Bacillus coli commune*. Centralbl. f. Bakteriol., **16**, 817.

MATTHEWS, A. P., AND WALKER, S. 1909 The spontaneous oxidation of cystein. Jour. Biol. Chem. **6**, 21.

MAYMONE, B. 1916 Sulla diagnosi differenziale dei paratifi tipo A e B. Archiv. per le scienze mediche, Torino, **40**, 202.

MEAD, L. D., AND GIES, W. J. 1901 Physiological and toxicological effects of tellurium compounds with a special study of their influence on nutrition. Amer. Jour. Physiol., **5**, 104.

MEUSEL, E. 1875 Nitritbildung duch Bakterien. Berichte des deutschen chemischen Gesellschaft **8**, 1214 and 1653.

MEYERS, JOHN T. 1920 The production of hydrogen sulfid by bacteria. Jour. Bacteriol., **5**, 231.

NĚMEC, A., AND KÁS, V. 1921 Ueber die Einfluss des Selens auf die Entwickelung einiger Scheimmelpilze aus der Gattüng Penicillicum. Biochem. Zeitschr., **114**, 12.

NENCKI. 1878 Uber den chemischen Mechanismus der Fäulnis. Jour. f. praktische Chemie, **17**, 105.

NEUBERG, C. 1914 Das Verhalten der α-Ketosäuren zu Mikroorganismen. I Mitteilung. Die Fäulnis von Brenztraubensäure und Oxalessigsäure. Biochem. Zeitschr., **67**, 90.

NEUBERG, C. 1914 Das Verhalten der α-Ketosäuren zu Mikroorganism. II Mitteilung. Die Faulnis von α-Ketobuttersäure. Biochem. Zeitschr., **67**, 122.

NEUBERG, C., AND GROSSER 1905 Eine neue schwefelhaltige Substanz aus dem Hundeharn. Centralbl. f. Physiol., **19**, 316.

NEUBERG, C., AND NORD, F. F. 1919 Anwendungen der Abfangmethode auf die Bakteriengärungen I. Acetaldehyde als Zwischinstufie bei der Vergärung von Zucker, Mannit und glycerin durch *Bacterium coli*, u. s. w. Biochem. Zeitschr., **96**, 133.

NEUBERG, C., AND NORD, F. F. 1919 Anwendungen die Abfangmethode auf die Bakteriengärungen. II Festleguuing den Aldehydestufie bei der Essiggärung. Biochem. Zeitschr., **96**, 158.

NEUBERG, C., AND REWALD, B. 1915 Das Verhalten der α-Ketosäuren zu Mikroorganismen. III Die Faulnis der d, l-methyl ethyl Brenztraubensäure. Biochem. Zeitschr., **71,** 122.

NEUBERG, C., AND ROSENBERG, E. 1907 Ueber die bei der Eiweissfäulnis auftretenden Fettsäuren sowie uber die optisch aktive Valeriansäure und Kapronsäure. Biochem. Zeitschr., **7,** 178.

PAKES, W. C. C., AND JOLLYMAN, W. H. 1901 Bacterial decomposition of formic acid into carbon dioxid and hydrogen. Jour. London Chemical Society **79,** 386.

PETRIE, R. J., AND MAASSEN, A. 1893 Beiträge zur Biologie der krankheitserregenden. Bakterien insbesonderes über die Bildung Von Schwefelwasserstoff durch dieselben unter vornehmlicher Berücksichtigung des Schweinerothlaufs. Arbeit. aus dem kaiserlichen Gesundheitsamte, **8,** 318.

PETRIE, R. J., AND MAASSEN, A. 1893 Weitere Beitrage zur Schwefelwasserstoff bildung aërober Bakterien und kurze Angaben uber Merkaptanbildung derselben. Arbeit. aus dem kaiserlichen Gesundheitsamte **8,** 490.

PETRUSCKY, J. 1889 Bakteriochemische Untersuchungen. Centralbl. f. Bakteriol. **6,** 63, 625 and 657.

PLIMMER, R. H. 1918 Citation in reference to coprosterol. Pract. Organic and Physiol. Chem., p. 34.

POEHL, A. 1886 Ueber einige biologisch-chemische Eigenschaften der Mikroorganismen in Allgemeinen und uber die Bildung der Ptomaine durch die Cholerabacillen in Speciellen. Berichte der deutschen chemischen Gesellschaft, **19,** 1159.

POHL, J. 1904 Ueber eine Alkylsynthese nach Thioharnstoffaufnahme. Archiv f. exper. Pathol. u. Pharmakol., **51,** 341.

QUARELLI, G. 1913 Sulla chemoterapia col selenio. Pensiero medico, **3,** 209.

RABUTEAU. 1869 Recherches sur les propriétés et sur l'élimination des composés oxygénés du sélénium et du tellure. Gazette hebdomodaire de médicin et de chirurgie, **6,** 194 and 241.

DE REY-PAILHADE, J. 1894 Études sur les propriétés chimique de l'extrait alcoolique de levûre de bière; formation de l'acide carbonique et absorption d'oxygène. Compt. rend. des séances de l'académie des sciences, **118,** 201.

DE REY-PAILHADE, J. 1888 Bulletin de la société chimique, **106,** 1683; 1890, 171.

REDFIELD. 1912 Hydrogen sulfid production and its significance in the sanitary examination of water. Ph. D. Thesis, Cornell.

ROSENHEIM, O. 1902 The decomposition of the compounds of selenium and tellurium by molds and its influence on the biologic test for arsenic. Proc. Chem. Soc. London, **18,** 138.

ROSZAHEGYI, A. 1898, 1899 Ueber das Züchten von Bakterien in gefärbter Nährgelatine. Centralbl. f. Bakteriol., **24,** 513; **25,** 15, also 69.

SALKOWSKI, E., AND H. 1883 Ueber die Entstehung der Homologender Benzoesäure bei der Fäulnis. Zeitschr. f. physiol. Chemie, **7,** 450.

SALKOWSKI, E., AND H. 1883 Zur Kenntnis der Eisweissfäulnis. II Die Skatolcarbonsäure. Zeitschr. f. physiol. Chemie, **9,** 8.

SALTET, R. H. 1900 Ueber Reduktion von Sulfaten in Brackwasser durch Bakterien. Centralbl. f. Bakteriol., II Abt., **6**, 648, 695.

SENATOR. 1918 Citation in reference to auto-intoxication. Wells' Chemical Pathology, 3rd edition, p. 581.

SCHEUERLEN. 1900 Die Verwendung der selenigen säure in der Bakteriologie. Zeitschr. f. Hyg. u. Infektionskrankh., **33**, 135.

SCHARDINGER, F. 1902 Ueber das Verhalten der Kuhmilch gegen Methylenblau Zeitschrift für Untersuchung der Nährung-und Genussmittel., **5**, 1113.

SCHARDINGER, F. 1894 Beitrage zur hygienischen Beurteilung des Trinkwassers. Centralbl. f. Bakteriol., **16**, 853.

SMITH, TH. 1896 Reduktionserscheinungen bei Bakterien und ihre Beziehung über Reduktionserscheinungen in steriler Bouillon. Centralbl. f. Bakteriol., **19**, 181.

SOMMARUGA, E. 1892 Ueber Stoffwechselproducte von Mikroorganismen. Zeitschr. f. Hyg. u. Infektionskrankh., **12**, 273.

SPINA, A. 1887 Bakteriologische Versuche mit gefärbten Nährsubstanzen. Centralbl. f. Bakteriol., **2**, 71.

STEPANOW, A. 1902 Ueber die Zersetzung des Jodkalium im Organismus durch Nitrite. Archiv. f. exper. Pathol. **47**, 411.

STOKVIS, C. S. 1899 Bijdrage tot verklaring der Zeavelwaterstofvorming in het Amsterdamsche grachwater. (Academisch proefschrift).

TANNER, F. W. 1917 Studies of the bacterial metabolism of sulfur. Formation of hydrogen sulfid from certain sulfur compounds under aerobic conditions. Jour. Bacteriol., **2**, 585.

THUNBERG, T. 1917 Die biologische Bedeutung der Sulfhydrylgrippe. Ergebnisse der Physiologie **11**, 328.

THUNBERG, T. 1920 Zur Kenntniss des intermediären Stoffwechsels und der dabei wirksamen Enzyme. Skandinavisches Archiv für Physiologie **40**, 1.

UFFELMAN, J. 1891 Ueber den Nachweiss der Typhusbacillen. Berlin. klin. Wochenschr. **28**, 857.

VOHL 1865 Citation from Eulenberg, H. Die Lehre von den schädlichen und giftigen Gasen, p. 454.

WICHERN, H. 1910 Quantitative Untersuchungen ueber die Reduktionswirkung der Typhus-Coli Gruppe. Archiv f. Hyg., **72**, 1.

WIELAND, H. 1914 Ueber den Mechanismus der Oxydationsvorgänge. III Berichte der deutschen Chemischen Gesellschaft, **47**, 2085.

WÖHLER, F. 1840 Telluraethyl. Annalen der Chemie und Pharmacie, **35**, 11.

WOLFF, K. 1899 Ueber Dentrifikation. Hygienische Rundschau, **9**, 546.

WOLFF, J. 1909 Sur quelques propriétés nouvelles des oxydases de Russala Delica. Compt. rend. des séances de l'académie des sciences, **148**, 500.

WODDRUFF, I. O., AND GIES, W. J. 1902 On the toxicology of selenium and its compounds. Amer. Jour. Physiol. Proc. of the Amer. Physiol. Soc., **6**, 29.

WROBLEWSKI, A. 1898 Zusammensetzung des Buchner'schen Hefepressaftes. Berichte der deutschen chemischen Gesellschaft, **31**, 3218.

WURTZ 1891 Bacille d'Eberth et coli-bacille. Le bulletin médical **5**, 1155.

PLATE 1

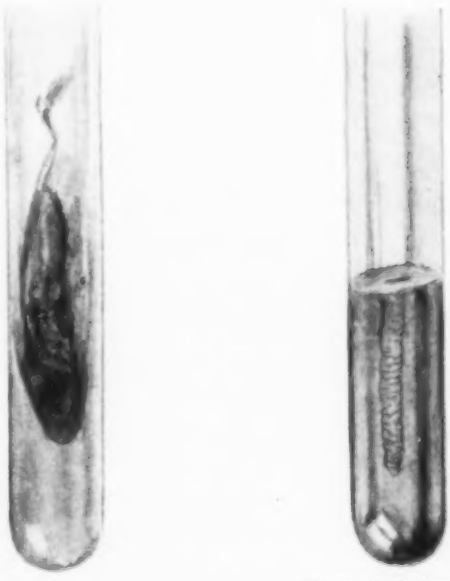
ILLUSTRATIONS OF BIOLOGICAL REDUCTIONS OF SELENIUM COMPOUNDS

FIG. 1. *B. coli* grown on a sodium selenite culture medium. The colonies appear as red spots.

FIG. 2. Another culture of *B. coli* grown on a slant.

FIG. 3. Section of the liver of a dog that had been subcutaneously treated with 2 mgm. selenium dioxid per kilo of body weight. The chocolate red granules represent deposited selenium. The cells, especially the nuclei, have suffered degeneration to the extent that they no longer stain readily.

FIG. 4. Stab culture of *Streptococcus pyogenes*.



(Levine: Reducing properties of microörganisms)

STUDIES ON CERTAIN CHARACTERISTICS OF CLOSTRIDIUM CHAUVEI AND CLOSTRIDIUM EDEMATIS[1]

JOSEPH P. SCOTT

Received for publication July 26, 1924

INTRODUCTION

A knowledge of the exact biological factors involved in blackleg and blackleg-like diseases of cattle and other animals is essential in order to devise methods for controlling these diseases. The following experiments relating to cultural characteristics, pathogenicity, production of aggressive substances, immunization, and serological tests have been made in order to determine certain characteristics that may serve to differentiate *Clostridium chauvei* from allied anaerobic spore-forming rods. In these experiments thirty-six strains of *Clostridium chauvei* have been used for the more important tests, seventeen in the cultural tests and a smaller series for some of the less important determinations.

HISTORICAL RÉSUMÉ

In 1782 Chabert differentiated between anthrax and blackleg on symptomatic and pathological grounds. The causative agent of blackleg was described by Bollinger (1875) and Feser (1876). Pasteur, who by the isolation of *Bacillus butyricus* in 1861 opened up the field of anaerobic bacteriology, in 1877 isolated (Pasteur 1877) a pathogenic anaerobe, the *Vibrion septique*, from the blood of a horse and a cow. In the following year Arloing, Cornevin and Thomas (1876) reported on the artificial cultivation of *Bacterium chauvei* in chicken broth containing either glycerol

[1] Contribution number 30 from the Veterinary Department of the Kansas Agricultural Experiment Station. Prepared under the direction of Dr. L. D. Bushnell of the Department of Bacteriology, as a thesis for the degree of M.S. at the Kansas State Agricultural College.

or iron sulphate. Koch (1886) grew the *Rauschbrandbaccillus* on gelatine and potato. Liborius (1886) simplified the study of anaerobes and described eight methods of isolating and growing these organisms in pure culture. Roux (1887) used a method of growing anaerobes in sealed flasks containing slightly alkaline broth. Later he and Chamberland (1887) isolated toxin from pure cultures of the *Vibrion septique*. This toxin, when used in doses up to 40 cc. and given in two doses, produced an active immunity in guinea-pigs. Filtered muscle juices and tissue exudates proved to be more toxic, but could be used in immunization if the dosage was graduated. The following year Roux (1888) found that toxins could be produced from filtered cultures of *C. chauvei* and from tissue exudates of blackleg cases. He found that guinea-pigs immunized with these toxins or with attenuated pure cultures of the *Vibrion septique* were immune to the *Vibrion septique* but not to blackleg. Guinea-pigs similarly immunized against blackleg were immunized against both diseases.

Kitasato (1889) found that the addition of glycerol or of 1 to 2 per cent glucose to broth did not increase the growth of the blackleg organism. He used solid and liquid media to which he added fresh guinea-pig tissue. He was able to immunize guinea-pigs by the use of old and attenuated broth cultures of *C. chauvei*. The young of female guinea-pigs vaccinated while pregnant proved to be immune up to the age of fifty days. In 1890 Kitasato and Weyl used various reducing substances incorporated in agar and broth for the cultivation of anaerobes (Kitasato and Weyl, 1890). In a second paper (1890) Kitasato described the blackleg organism as a single rod and occasional pairs forming an acute angle one with another. He found that sporulation did not occur until some hours after death.

Leclainchee and Valée (1900) recognized the cultural resemblances between the *Vibrion septique* and *C. chauvei*. They differentiated these organisms by the agglutination test and by the use of actively and passively immunized guinea-pigs. They also produced a very virulent toxin from broth cultures of *C. chauvei*.

Grassberger (1902) described two types of the blackleg organism, one non-motile and non-spore-forming, the other motile and sporulating. Shattenfroh (1903) found numerous intermediate types between the *Vibrion septique* and blackleg. In 1904 Grassberger and Shattenfroh produced a toxin from pure cultures of the *Rauschbrandbaccillus*. They found that different strains had to be given different substances for the best production of the toxin, either glucose or calcium lactate having to be supplied. They (Grassberger and Shattenfroh 1907) also produced an antitoxin by immunizing animals with this toxin. This antitoxin was used in differentiating blackleg from other diseases. It was found that tissue extracts also contained a toxin and they described two groups of *C. chauvei*, (1) a highly virulent non-toxic group and (2) a slightly virulent toxic group.

Werner (1905) differentiated the gas gangrene organisms by means of the agglutination test, using agar cultures grown in an atmosphere of hydrogen, both for immunization and as the antigen in the test.

Eisenberg (1907) produced a very toxic product by decantation and centrifugation of six-day broth cultures of *C. chauvei*. Doses of 0.005 cc. of the toxin killed guinea-pigs in a few minutes. Pfuhl (1917) described a method of growing anaerobes aerobically in liver broth. He found that liver broth absorbed oxygen and produced carbon dioxide. He reviewed the previous attempts to grow anaerobes aerobically, those of Catoni (1891) who grew the tetanus bacillus in rabbit blood, of Tarrozzi and Wryosek (1905) who used nutrient broth containing pieces of fresh meat and of Harass (1906) who used liverbrew-liverbroth and brainbrew-brainbroth media.

Von Hibler (1908) described the characteristics of fifteen anaerobic species, noting their morphological and cultural characters in detail. These organisms were grouped by means of the deep agar colony formation, the blackening of brain medium and their proteolytic characters. He found that *B. welchii*, *B. sporogenes*, blackleg and pseudo-blackleg organisms formed smooth colonies, the other anaerobes irregular or woolly colonies.

Balavaine (1909) discussed the results of vaccinating cattle

against blackleg. He found that vaccination might cause death from blackleg or might fail to give lasting immunity.

Foth (1909-1910) discussed the differentiation of blackleg from anthrax and from other anaerobic diseases; using the agglutination reaction he described *C. chauvei* as a single rod, while most other organisms found in similar diseases were chain-forming rods. He found that the characteristic chains or single rods were formed on the diaphragmatic surface of the liver.

Hasenkamp (1909) described a case of blackleg in a horse, diagnosing the condition after finding numerous blackleg organisms in smears from the affected tissues.

Schöbl (1919) described a method of immunizing guinea-pigs by the use of filtered blackleg exudates, or aggressins.

MacCrudden (1910) studied the proteolytic properties of *C. chauvei* and *Clostridium salus*, finding that *C. salus* produced gas, tyrosin, leucin, and trytophane, while *C. chauvei* produced little gas and tryptophane. Neither produced indol and skatol. Veillon and Masé (1910) found that the addition of potassium nitrate to agar facillitated the Veillon and Zuber deep agar colony method of isolation.

Markoff (1911) compared the organisms isolated from typical cases of blackleg with parturient blackleg and also with some chain formers. He found that the blackleg group are pathogenic for old guinea-pigs only, parturient blackleg types for both young and old guinea-pigs, and the chain formers for all animals. Differentiation could be made by means of the guinea-pig lesions, stained liver smears, toxin production, and the agglutination test. He found it possible to use the microscopic agglutination test in the examination of impure or mixed cultures.

Diederichs(1911) reported on two cases of disease in horses. One was diagnosed as pseudo-blackleg, since the organism isolated was pathogenic for guinea pigs and rabbits, the other as true blackleg, since the organism in this case was pathogenic for guinea-pigs only.

Möller (1911) found that the glycogenic content of muscles determined the extent of the lesions produced. He found that guinea-pigs fed on sugar beets developed very extensive lesions

when injected with blackleg virus, while starved animals developed only slight lesions or were refractory to the inoculation.

Wulff (1912) used the agglutination reaction to differentiate blackleg from other conditions. He disproved the theory that blackleg is a wound infection by demonstrating (1) that there are numerous cases of blackleg showing no muscle or subcutaneous lesions, and (2) that blackleg occurs when there are neither macroscopic nor microscopic wounds. In a second paper (1912) he showed that both blackleg and malignant edema organisms may be found in the bile, thus disproving von Hibler's observation on malignant edema and showing that the presence or absence of organisms in the bile is of no value for diagnostic purposes.

Hölzel (1913) grew *C. chauvei* aerobically in broth containing starch.

Lechlainchee and Valée (1913) reported on the production and use of a pure culture blackleg vaccine obtained by growing cultures at 42°C.

Grassberger and Shattenfroh (1913) stated that biological reactions are of secondary importance in the differentiation of blackleg from other diseases and that the most reliable test is the production of the toxin by the culture and the use of blackleg antitoxin to test its specificity.

Detre (1913) immunized horses by the use of pure cultures of *C. chauvei*. He showed that the serum contained the specific agglutinins and that doses of 0.005 to 0.02 cc. protected guinea-pigs.

Von Ratz (1913) found that swine were susceptible to blackleg.

Mieszner (1913) used the Abderhalden dialysis reaction for the differentiation of blackleg.

Lesage and Pommier (1913) reported on an outbreak of blackleg in sheep following lambing.

Hecht (1913) used the Ascoli thermo-precipitin reaction for the diagnosis of blackleg.

Köves (1914) studied the so called symptomatic anthrax of swine and isolated a typical Ghon-Sachs organism from the intestinal lesions. This organism produced typical muscle lesions

in inoculated hogs. Later (1918) he studied this disease in detail and compared the organisms isolated with *C. chauvei* and malignant edema cultures.

Nicolle, Cesari, and Raphael (1915) experimented with the toxins of *C. chauvei* and the *Vibrion septique* and found that the toxicity was greatly reduced at 55 degrees. They stated that the cultural and serological differences between these two organisms are slight.

Meyer (1915) isolated the Ghon-Sachs organism from symptomatic anthrax of swine.

Von Wasserman (1916) compared the gas gangrene organism with *C. chauvei*. He found that the intestinal types of gas gangrene and blackleg or bradsot were very similar.

Richter (1916) showed that parturient blackleg of cattle and blackleg of horses are mixed infections in which malignant edema organisms can usually be found. *C. chauvei* also may be found in some cases. He also described a septicemic type of true blackleg.

Conradi and Beiling (1916) used the agglutination test to divide the malignant edema organisms into two groups, these groups being transformable from one into the other by proper cultural methods.

Franklin and Haslam (1916) showed that blackleg powder and pellet vaccines are unsatisfactory. Later (1920) they reported on the isolation of a pseudo-blackleg organism from some of this material.

Goss (1917) reported on the production of blackleg aggressin and anti-serum, and later (1919) on the production of blackleg filtrate. In the following year he and Scott (1918) reported on the potency tests for these products and described a potency test based on aggressive action.

Todd (1917) reported on the gas production of *C. chauvei*, *B. edematis-maligni* Koch, and other anaerobes.

Warnecke (1917) described a disease in a young calf which resembled blackleg but from which he isolated an organism resembling *Bact. coli*.

Fürth (1917) isolated a diplobacillus from cases of human

gangrene; he agglutinated this organism by blackleg anti-sera, and identified it as *C. chauvei*.

Landau (1917) found that the results from the agglutination test and from protection tests with blackleg and malignant edema anti-sera do not coincide.

Frommeld (1917) found that blackleg cultures containing spores were killed by exposures to thirty hours of direct sunlight.

Bohler (1917) described a case of blackleg in a horse suffering from shrapnel wounds.

Jensen (1917) described 10 cases of bradshot in sheep. He found characteristic intestinal ulcers from which the organism was isolated. Feeding experiments with these cultures gave negative results. He suggested that the cadaver bacillus which was also found in the diseased animals played a part in the infection. Histological studies proved that the organism entered through the digestive tract.

Foth (1918) described the use of a new blackleg vaccine "Emphysarcol," consisting of an attenuated suspension of spores and a spore-free filtrate. The attenuated spores are injected into the tail, the filtrate into the ear. Kelser (1918) discussed the immunity produced by blackleg filtrates. Berg (1918) described a method of concentrating this toxin in vacuo.

Pfeiler (1918) used the precipitin reaction for the diagnosis of anthrax, blackleg, swinepest and other diseases.

Szasz (1918) found it inadvisable to vaccinate cattle against anthrax and blackleg at the same time. Blackleg vaccination should be given fourteen days after the anthrax vaccination.

Schmitt (1918) diagnosed as blackleg a disease in a foal from which he isolated an anaerobe. This anaerobe occurred as a single rod or in pairs forming an angle one with another.

Nitta (1918) described the Japanese method for producing blackleg filtrate.

Steinbrucke (1918) stated that the gas gangrene organisms belong to the blackleg group.

Weinberg and Séguin (1918) discussed the etiological factors of gas gangrene and clarified the classification of malignant edema organisms and the *Vibrion septique*. They did not find *C. chauvei* in war wounds.

Van Heelsbergen (1919) found that the etiological factors of gas gangrene, malignant edema and blackleg were very similar. He thought that they should be classified under one species as Grassberger and Shattenfroh had suggested. Haslam and Lumb (1919) showed that the toxicity of filtrates had no relationship to their potency.

Zeiszler (1919) showed that spontaneous blackleg, parturient blackleg, whale septicemia and reindeer pest are all primarily caused by the blackleg organism. He isolated *C. chauvei* from a few cases of human gas gangrene and found that the agglutination reaction was unreliable for the differential diagnosis of anaerobic organisms.

Ravenna (1920) described the cardiac, pulmonary and arterial lesions of blackleg and showed the disease to be a septicemia.

Graub and Zschokke (1920) found that germ-free filtrates protected cattle against 2 M.L.D. virus. The immunity produced by these filtrates was greatly increased by the use of an attenuated virus given ten days after the filtrate.

Speigl (1920) found that blackleg in sheep might be due to Foth's blackleg organism, to the Ghon-Sachs organism or to *B. welchii*.

Haslam (1920) tabulated the results following the use of blackleg aggressin. He described the methods for testing the sterility of this product and found that brain liver medium inoculations were much more reliable than animal inoculation.

Ronca (1920) observed that the digestive juices do not injure *C. chauvei*. He produced blackleg in guinea-pigs by feeding very virulent cultures. Infection was regularly produced if the organism was introduced into a loop of the intestine. In animals infected by way of the digestive tract the characteristic lesions were found in certain parts of the body.

Heller (1920) discussed the etiological factors of blackleg and allied anaerobic diseases. She found that cattle are very susceptible to infection by the blackleg group and are occasionally affected with the *Vibrion septique* group; sheep, horses, and swine are usually affected by the *Vibrion septique* group; sheep occasionally by the *Chauvei* group and horses sometimes by

the *Oedematiens* group. She found *B. welchii* infections in animals very rare.

Gochenour (1920) used the complement fixation reaction for the identification of *C. botulinus* in canned foods.

Goss, Barbarin and Haines (1921) found that *C. chauvei* does not ferment carbohydrates. They used the passive immunity produced by anti-blackleg serum as a means of differentiating the pathogenic anaerobes. They found that anti-blackleg serum agglutinated blackleg strains in dilutions of 1:800 and *Vibrion septique* strains in dilutions of 1:200.

Ronca (1921) studied the changes in the blood pictures of animals injected with blackleg virus and toxin. He found first a decided increase followed by a progressive decrease in certain types of leucocytes in animals that died; in animals that recovered, the decrease was followed by an increase in both leucocytes and erythrocytes.

Scott (1921) described the methods of producing anti-blackleg serum and showed that the potency of this product did not decrease for several years.

Hall (1922) described the various species of spore-bearing anaerobic rods. He used spore formation as the primary criterion of classification. He found that *C. chauvei* ferments sucrose but that the *Vibrion septique* does not.

Scott (1922) described the production of blackleg anti-serum, blackleg aggressin and blackleg filtrate, and later (1923) reported on potency tests for aggressin and filtrate based on the aggressive action of these products.

Leclainchee and Valée (1923) supplemented their previous work on toxins. They reported on the production of a toxin from an extract of cells obtained from a twenty-four hour culture of *C. chauvei* and on a non toxic extract made from cells in a six day culture.

Göertiller (1923) stated that both cultural and immunological tests are necessary for the proper identification of anaerobes. He found that the most satisfactory tests were the toxin-antitoxin test and the immunization of guinea-pigs.

Reddish and Rettger (1924) described several sporulating

anaerobes. Their type *C. chauvei* strain produced deep agar colonies having a dense nucleus and radiating projections. In culture media short chains were found to be not uncommon.

HISTORY OF CULTURES USED

In the course of this work 35 strains of *C. chauvei* and 5 strains of *Clostridium edematis*[2] have been studied.

C. chauvei strains

Strains 1 to 10 were isolated between 1911 and 1916 by Drs. O. M. Franklin and T. P. Haslam from blackleg material obtained from field cases in Kansas and other states. These ten strains were used to produce the serials of blackleg anti-serum which were used in the serological tests. Strains 3, 4, 8 and 9 were discarded between 1921 and 1923 due to decreased virulence or to contamination.

Strain 11 was isolated from dried muscle obtained from a case of blackleg in 1917. It was discarded in 1919. Strain 12 was isolated in 1917 from blackleg muscle. Strain 14 was obtained in 1918 by Dr. L. W. Goss of the Kansas experiment station from a vial of liquid blackleg vaccine purchased from the Pasteur Laboratories; it was discarded in 1921. Strain 15 was isolated in 1918 by Dr. L. W. Goss from dried blackleg muscle sent from the Bureau of Animal Industry, United States Department of Agriculture, Washington, D. C. It was discarded in 1921.

Strain 16 was isolated in 1918 by Dr. Goss from blackleg tissue obtained from a field case of blackleg in southern Kansas. This strain was discarded in 1920 due to decreased virulence.

Strain 17 was obtained in 1918 from the Continental Serum Company. It was discarded in 1921.

[2] The term *Clostridium edematis* has been used in this paper instead of *Vibrion septique*, in accordance with the report of the Committee of the Society of American Bacteriologists. Jour. Bact., vol. 5, no. 3, p. 22, 1920. Bergey in Bergey's Manual of Determinative Bacteriology, 1923, p. 325, uses the term *Clostridium oedematis-maligni* presumably to avoid confusion with *clostridium Cedematiens*.

Strain 18 was isolated in 1918 by Dr. Goss from a case of blackleg. It was discarded in 1921.

Strain 19 was obtained in 1919 from Dr. Haslam of the Purity Biological Company.

Strain 20 was isolated in 1919 by Dr. Goss from material obtained from a case of blackleg in Riley county. This strain was discarded in 1921.

Strain 22 was obtained in 1919 from a vial of liquid blackleg vaccine obtained from the Jensen Salsbery Laboratories. It was discarded in 1921.

Strain 23 was isolated in 1921 from blackleg material taken from a calf inoculated with culture virus strain 1.

Strain 24 was obtained as a pure culture from Parke, Davis and Company in 1921.

Strain 25 was obtained as a pure culture from Miss H. H. Heller of the Hooper Foundation, San Francisco. This strain was discarded in 1921.

Strain 26 was isolated in 1921 from a field case of blackleg; it was discarded in the fall of 1921.

Strain 27 was isolated in 1922 from a calf inoculated with culture 21.

Strain 28 was isolated in 1922 from dried blackleg muscle obtained from the Mulford Company.

Strain 29 was isolated in 1922 from dried muscle tissue from a case of blackleg sent in for diagnosis.

Strain 30 was obtained in 1922 from blackleg material sent from the Mulford Company.

Strain 31 was isolated in 1922 from a case of blackleg in a yearling calf in Riley county, Kansas.

Strain 32 was isolated in 1922 from muscle tissue sent in for diagnosis from a case of blackleg in Wakefield, Kansas.

Strain 33 was obtained as pure culture from Prof. I. C. Hall of the University of California, Berkeley, California.

Strains 34 and 35 were isolated from an outbreak of blackleg in a herd of purebred cattle in Riley county, Kansas.

Strain 36 was isolated in 1924 from a case of blackleg treated in the ambulatory clinic.

Clostridium edematis strains

Strains C.E. 1, C.E. 2 and C.E. 3 were obtained from the Lister Institute, London. These strains were labeled *Vibrion septique* types, 1, 2, and 3.

Strain C.E. 4 was obtained from Prof. I. C. Hall of the University of California.

Strain C.E. 5 was isolated in 1922 from a case of malignant edema or so-called "symptomatic anthrax" in a hog.

CRITERIA USED TO DETERMINE THE PURITY OF C. CHAUVEI STRAINS

Up to 1920 cultural and morphological reactions were considered a sufficient indication of purity. At that time, after isolation of the strain by means of the deep agar colony method, eight cultural and morphological reactions were introduced.

1. In deep agar only those strains producing small spherical or lenticular colonies with definite solid borders were considered as typical.

2. In brain liver medium typical cultures showed cloudiness and gas production in from two to four days, after which the fluid portion rapidly cleared due to sedimentation of the organisms and spores. The brain never showed any darkening and the broth, if dark on inoculation, became amber colored on clearing.

3. The culture when freshly isolated must be pathogenic for guinea-pigs. Since 1920 cultures that have lost their virulence have not been discarded but are still used for production of filtrate. Typical edematous swellings must be produced in guinea-pigs around the point of inoculation and death must follow within three days.

4. The typical single Gram-positive rods with rounded ends must be found both in culture smears and in impression preparations made from the peritoneal surface of the liver of inoculated guinea-pigs.

5. The typical blackleg odor should be obtained both from the culture media and from guinea-pigs inoculated with the culture. However, some strains of *C. chauvei* produce no noticeable odor either in guinea-pigs or in culture media.

6. Guinea-pigs immunized by inoculation of 7 units of anti-blackleg serum must withstand the administration of 5 M.L.D. of the culture, given fifteen hours after the serum.

7. Inspissated blood serum must show no changes when inoculated and incubated anaerobically for five to seven days.

8. No growth may develop in 2 per cent glucose beef infusion agar cultures.

PATHOLOGICAL LESIONS

Blackleg is an acute, infectious, but non-contagious disease of cattle, and exceptionally of other ruminants. Young cattle from six months to two years of age are found to be most susceptible. The disease is characterized by a sudden appearance of lameness, and gas production in the affected tissues, followed by prostration and death in from twelve to thirty-six hours.

The lesions produced in calves are essentially muscular and subcutaneous in character. On opening the animal the characteristic blackleg odor is first noticed and extensive subcutaneous lesions containing dark-red exudate and gas may be found. The muscles of one of the quarters are found to be greatly distended, due to accumulation of numerous small gas bubbles throughout the muscle tissue. Areas and streaks of blackened muscle are found, intermixed with lighter pink areas, giving the muscle a characteristic mottled appearance. Some muscles show no black spots although filled with gas bubbles and of a pink color. The lesions have a tendency to be limited to single muscles and do not pass through the intermuscular septa. The internal organs are fairly normal in a fresh carcass. The serous membranes are injected and a black clot is found in the heart. No marked gas formation is noticed in the intestines. The peritoneal and pleural cavities contain large amounts of a dark-red exudate.

The disease in guinea-pigs inoculated with blackleg material in the axillary space follows a characteristic course. There is first a clearly defined edematous swelling developed around the point of inoculation, which extends to the pelvis in from thirty-six to seventy-two hours. The height of the swelling is from 2 to 4 mm. If death does not take place the hair over the

lesion is shed and the skin along the median line usually opens disclosing a line of blackened muscle. If death occurs, and the animal be opened, the subcutaneous tissue are found to be full of a red exudate; there is some gas in the muscles and they are either black or pinkish in color. The serous membranes are injected, and the blood in the heart is black in color. No gas is found in the intestines unless the animal has been dead for more than twelve hours.

MODE OF ENTRANCE OF THE ORGANISM

It has usually been considered that *C. chauvei* enters the body by means of small skin abrasions. Since 1912 considerable work has been done based on this supposition. It has been shown that the infection can enter through the digestive tract, but no evidence has been brought forward to show that the organism ever enters by way of the skin.

Wulff (1912) discounted the wound infection theory on two grounds: (1) That there were numerous cases of septicemic blackleg in which no muscles lesions were found, but from which the blackleg organism was isolated, and (2) he was unable to find any case in which skin wounds could be demonstrated, although he examined hundreds of microscopic sections from blackleg lesions. In confirmation of this the following case may be cited. In the author's work, during the last year, one case of the septicemic form of blackleg was encountered in a steer eight months of age which had been vaccinated at five months of age. It was noticed to be off feed one evening; next morning it was slightly lame and was dead by noon. When examined, the carcass was very badly distended with gas; no muscle lesions could be found; the serous membranes were injected; the heart contained a black clot; and the characteristic blackleg odor was very noticeable. A pure culture of *C. chauvei* (strain 37) was isolated from this case together with an unidentified anaerobe whose connection with the disease has not yet been demonstrated. Several other cases of the septicemic type have been reported to us from material sent to the laboratory for diagnosis. From some of this material *C. chauvei* has been isolated.

Ronca (1920) produced blackleg in guinea-pigs by feeding virulent cultures and also by introducing cultures into the intestinal canal.

In a recent outbreak in Kansas the infection was traced to pigs that had fed on the carcass of an animal that had died of blackleg. These pigs walked through the manger of some steers in an isolated lot. Some of these animals developed blackleg within three days. In another case a yearling steer developed a hard swelling of the brisket within twelve hours lameness in the right hind leg developed, followed by a swelling in the same quarter and death from blackleg in forty-eight hours. This case showed that the primary lesion may be located at a considerable distance from the final muscle lesion and also that blackleg is not a true wound infection, which presupposes greater or less destruction of tissue in which the organism must develop.

In the case of the similar disease "malignant edema" of swine or so-called "symptomatic anthrax." Koves (1914) showed that intestinal lesions were produced from which the causative organism could be isolated. This organism produced the typical black muscle lesions in other swine.

ISOLATION

All strains studied have been isolated by (1) the cultural method or (2) by the guinea-pig inoculation method.

In the cultural method brain liver medium is inoculated with some of the material to be isolated, and is then heated to between 65° and 70°C. and held at this temperature for fifteen minutes. A check tube is also inoculated from unheated material, to be used in case the heated tubes prove to be sterile. After twenty-four hours incubation three or four serial dilutions are made, into freshly heated liver agar tubes at 45°C. Twenty four hours later typical colonies are picked off into brain liver medium. This is repeated once a day till at least four sets of agar dilutions have been made. The culture is then tested on agar slants for anaerobic contamination and is inoculated into a guinea-pig for preliminary identification tests.

At the same time that the brain-liver medium is inoculated,

a guinea-pig is injected with some of the material and the organism isolated from the muscle lesions and heart blood as soon as the pig dies. In both cases the strain is preferably reisolated from the test guinea-pig injected with the culture after preliminary isolation.

MORPHOLOGY

C. chauvei is a medium-sized rod with rounded ends. It is Gram-positive except in old cultures which may show Gram-negative rods. The organism is from four to six times as long as it is wide, 3 to 6 microns in length by 0.5 micron in width. In smears made from the peritoneal surface of the liver the Gram-positive rods are easily found. They are usually well scattered throughout the preparation, not more than six to ten organisms being found in any one field. Organisms containing oval sub-terminal spores may be found in preparations made from animals that have been dead for some time. Usually these spore-bearing rods are very scarce. Pairs, in which the organisms are end to end, may occasionally be found and very rarely chains of three or four elements may be seen. In slides made from culture media the picture is very similar. In young cultures spore-bearing rods are often absent. In older cultures free spores are usually present and these are frequently unstained. Paired organisms are perhaps even less common than in liver preparations. In slides made from *C. edematis* cultures there are usually a large number of rods in each field of which a relatively large number are spore-containing. Chains of two to six or more elements are fairly common. Some strains of *C. edematis*, notably type 2, usually occur in pairs in which the elements form an acute angle one with another. Chains in this strain are uncommon.

COLONY FORMATION

Surface and deep agar colony forms have been used by numerous authors as one of the important methods of differentiation between *C. chauvei* and other anaerobes.

Kitasato (1890) described the colonies formed by *C. chauvei* as having a central nucleus surrounded by numerous fine pro-

jections. Von Hibler (1908) described the deep agar colonies as lenticular or spherical in shape and having a smooth solid outline. Zeiszler (1920) describes two types of *Rauschbrand:* The surface agar colonies formed by the Kitt type being circular with a solid border or veil like, and the Foth type forming pearl button colonies having a raised center. Kitt, as quoted by Heller (1920), described the deep-agar colonies as fine, point shaped, or small, woolly spherical colonies. Heller found colonies with smooth outlines only. Goss et al. (1921) described the colonies of *C. chauvei* in 2 per cent agar as small, spherical or elliptical, and translucent, while colonies formed by *C. edematis* were woolly or fluffy. They state that in soft agar (one per cent) the colonies of *C. chauvei* may resemble those of *C. edematis.* Hall described the deep agar colonies as semi-transparent opaque spheres with or without raylike growths. Reddish and Rettger (1924) describe the deep agar colonies as being about 1 mm. in diameter and as having a solid nucleus surrounded by irregular projections radiating out in matted formation.

In 2 per cent liver agar all strains considered in this paper produced very small colonies, spherical or pin point in character. In some cases when the agar was considerably concentrated due to evaporation invisible colonies were formed and the only indication of growth was small gas bubble formation. In 1 to 1.5 per cent agar the colonies are larger and may be small circular discs or lenticular in shape. In all cases a regular smooth definite border has been noted. The colonies formed by the *C. edematis* strains studied were typically biconvex-lenticular in shape with the edges thin and flat. Outgrowths especially from the lower surface are common. In old colonies, double discs have been found. In soft agar markedly fluffy colonies are produced.

CULTURAL CHARACTERISTICS

Milk

Von Hibler (1908) found that *C. chauvei* produced a fine clot in forty-eight to ninety-six hours. Hall (1922) found that in milk containing sterile blood, coagulation may occur. Goss et al.

(1921) found that *C. chauvei* will not grow in litmus milk even when inoculated in large amounts, while *C. edematis* produces acid fairly readily. Reddish and Rettger (1924) reported that an acid clot is formed by both types.

None of the 35 strains considered in this paper produced any change in fresh, litmus, or brom-cresol-purple milk, when inoculated in amounts of from 1 to 6 loopfuls. However, when 1 mil of brain liver culture was used a slight thickening of the milk was noticed in some cases after forty-eight hours. *Clostridium edematis* strains produced coagulation in fresh milk when four to six loops of brain-liver culture were used as the inoculum.

Gelatin

Bacto gelatin containing 1 part glucose to 1000 was liquefied; while gelatin made from Gold Seal gelatin, even when made with liver broth or containing blood serum was not liquefied after two weeks' incubation in the anaerobic jar.

Coagulated serum

Coagulated serum is not affected by *C. chauvei* even after a month's incubation in the anaerobic jar.

Carbohydrate fermentation

The fermentation of carbohydrates has been studied by numerous workers. Nicolle, Cesari, and Raphael (1915) found no great differences between *C. chauvei* and *C. edematis*. Meyer (1915) also found practically no differences between the two types. However, the strain he described as *C. chauvei* (Munich) may be the same as the strain of *C. edematis* described by Hall (1922) as Meyer's *C. chauvei* Kitt, (Munich), in which case both organisms are to be considered as *C. edematis* strains. Goss et al. (1921) found that *C. chauvei* does not ferment any of the carbohydrates. These findings are shown in table 1. From table 1 it is seen that there is considerable difference in the results obtained. With the exception of salicin, in which all workers found no fermentation by *C. chauvei* and fermentation by *C. edematis*, there is a difference between at least one report and

TABLE 1

Carbohydrate fermentations as reported by various workers

CARBOHYDRATE	GOSS ET AL.		HALL		MED. RES. REPT. 39 ROBERTSON		REDDISH		PRESENT PAPER	
	Ch.	C.E.	Ch.	C.E.	Ch.	C.E.	Ch.	C.E.	Ch.	C.E.
Amygdalin	–	A					–	A G	F	F
Arabinose	–	A	–				A G	A G	F	F
Dextrine	–	A			–	–			–	F
Dulcitol					F	F	A G	A G	F	F
Galactose	–	A G	A G	A G	F	F	A G	A G	F	F
Glucose	–	A	–	–	–	–	A	–	F	F
Glycerol	–	A G					–	–		
Glycogen	–	A	–	–			–	–	F –	F
Inulin	–	A G			F	F	A G	A G	F	F
Lactose	–	A G	A G	A G	F	F	A G	A G	F	F
Levulose	–	A G	A G	A G	F	F	A G	A G	F	F
Maltose	–	A	–	–	–	–			–	F
Mannitol							–	A G	F –	F
Mannose	–	A					–	–	F	F
Raffinose							–	–	–	F
Rhamnose	–	A	A G	–	F	–	A G	–	F	– F
Sucrose	–	A G	–	A G	–	F	–	A G	–	F
Salicin	–	A					–	–	–	F
Sorbite							–	A G		
Soluble starch							–	–	–	F
Trehalose	–	A			–	–				
Xylose										
Amylum solution										

–, no fermentation; F, fermentation; A, acid; G, gas; –F, most strains do not ferment; F–, most strains ferment.
Medical Research Committee Report 39 as quoted by Heller.

the others. These conflicting reports may be due to differences in the strains studied, to the use of only a few strains, or to differences in the medium used as a base for the carbohydrates.

During the past four years various liquid and solid media have been used by us for the fermentation reactions of *C. chauvei*. The liquid media, with the exception of serum water, were made sugar-free in the ordinary way, brom-cresol-purple was then added and after sterilization 1 to 2 mils of 5 to 10 per cent carbohydrate solution added. The inoculated tubes were placed in a wire rack and held in the anaerobic pressure cooker. Anaerobic conditions were produced by burning phosphorus according to the method described by Bushnell (1922).

Sugar-free infusion broth prepared in the ordinary way and to which dextrine, galactose, glycogen, glucose, glycerol, inulin, lactose, maltose, mannitol, salicin, sucrose, or starch were added, was inoculated with one loopful of a twenty-four hour brain-liver culture, placed in the anaerobic cooker and incubated at 37°C. for ten days. Of ten strains so tested one produced acid and gas in glucose, lactose, maltose, and sucrose, all other tubes remaining unchanged.

Sugar-free liver broth: Four strains were tested on the same sugars using sugar-free liver broth as a base. No fermentation of any of the sugars was produced.

Serum water

The effect of six strains of *C. chauvei* and of strains 1, 2, 4 and 5 of *C. edematis* on the 20 carbohydrates listed in table 2 was determined. No fermentation of any of the carbohydrates was produced by any of the *C. chauvei* strains. All strains of *C. edematis* produced acid and caused coagulation of the serum so that gas production by these strains could not be recorded.

Serum water agar

Serum water agar was prepared by adding 20 grams of agar to 1000 mils Hiss serum water and proceeding with the ordinary method of agar preparation. The carbohydrate solutions were added while the agar was liquid and had been cooled to 40°C.

TABLE 2

Fermentation reactions of 17 strains of Clostridium chauvei and of five strains of Clostridium edematis in Serum Media

CARBOHYDRATE	1	3	5	6	7	10	12	19	23	24	27	28	29	30	31	32	33	C.E. 1	C.E. 2	C.E. 3	C.E. 4	C.E. 5
Arabinose	A G											A G					A	A G	A G	A G	A G	A G
Dextrine	A G											A G					–	A G	A G			
Dulcitol	–											–					–	A G	A G		A G	A G
Galactose	–	A G	A G	A G	A G	A G	A G	A G	–	A	A G	A G	A G	A G	A G	–	A G	A G	A G	A G	A G	A G
Glucose	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G
Glycerol	A G	A G	A G	A G	A G	A G	A G	A G	–	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G
Inulin	A G	A G	A G	–	A G	–	–	A G	–	A G	A G	A G	–	A G	A G	–	–	A G	A G	A G	A G	A G
Inositol	–											–					–	A G	A G		A G	A G
Lactose	–	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G
Levulose	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G
Maltose	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G
Mannitol	A G	A G	A G	–	–	–	A G	–	–	A G	A G	–	–	–	–	A G	A G	A G	A G	A G	A G	A G
Mannose	A G											A G					–	A G	A G	A G	A G	A G
Raffinose	A G											A G					A	A G	A G	A G	A G	A G
Rhamnose	–											–					–	A G	A G	A G	A G	A G
Sucrose	A G	A G	A G	A G	A G	A G	A G	A G	–	A G	A G	A G	–	A G	A	A G	A G	A G	A G	A G	–	A G
Salicin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A G	A G	A G	A G	A G
Starch	–											–					–	A G	A G	A G	A G	A G
Xylose	–											–					–	A G	A G	A G	A G	A G

A, acid production; G, gas production.

Fifteen strains of *C. chauvei* and the 12 carbohydrates listed in experiment 1 were used in this experiment. No fermentation was produced in any carbohydrate by any of the strains used. *C. edematis* strains also showed no fermentation in this medium but cultures of proteolytic anaerobes fermented most of the carbohydrates.

Sugar-free meat infusion or serum water agar plus sterile serum was found to be a very satisfactory medium for the study of fermentation reactions of both *C. chauvei* and *C. edematis*. Three sera were used (1) fresh and unfiltered serum, (2) fresh filtered serum, and (3) old filtered serum containing 0.05 per cent phenol. No marked differences were noticed in the fermentations produced in these three media.

Table 2 shows the results obtained from the fermentation tests on twenty sugars by seventeen strains of *C. chauvei* and five strains of *Clostridium edematis*. Some strains did not always give a consistent reaction and a negative reading has been recorded only when all tests showed a negative reaction.

It is seen that *C. chauvei* is much less saccharolytic than the *C. edematis* strains examined. Sucrose in one case was not fermented by *C. chauvei*. In all cases slight gas production was not counted as an evidence of fermentation unless acid was produced in the same tube.

PATHOGENICITY

Blackleg, although primarily a disease of cattle, has been reported in horses, swine and sheep. A disease of whales was reported by Nielson (1890) and by Christiansen (1920) from which an organism resembling, or identical with, *C. chauvei* was isolated.

The reports of the isolation of *C. chauvei* from horses are not conclusive. The diagnosis has usually been made on purely morphological grounds, or on morphological and guinea-pig and rabbit pathogenicity tests, as in the cases reported by Diederichs (1911). In the production of anti-blackleg serum normal horses were injected (Scott 1923) intravenously or subcutaneously with 25 to 30 mils of virulent cultures of *C. chauvei* without developing

any symptoms whatever. This must be considered conclusive evidence that horses are not susceptible to *C. chauvei.*

Reports of the isolation of *C. chauvei* from swine are occasionally met with in the literature. In these cases the diagnosis is also incomplete, being based on morphology, or on unstated cultural and other characters. On the other hand, all detailed experiments on the so-called "symptomatic anthrax" of swine (Koves, 1914 and 1918; Meyer, 1915) have shown that this disease which has symptoms very similar to blackleg is caused by a *C. edematis* type organism. This organism when injected into the muscles of hogs produces muscle lesions almost identical with those of blackleg in cattle. At the Kansas Agricultural Experiment Station two outbreaks of this disease in swine have been examined and from both a *C. edematis* type organism has been isolated.

One experiment on the susceptibility of swine has been made at the Kansas Station. A pig weighing 100 pounds was given 15 mils of virulent *C. chauvei* culture at the same time that a calf was given the same amount. The pig showed no rise in temperature and no lameness, while the calf died of blackleg within thirty-six hours. This experiment, taken together with the isolation of *C. edematis* strains in the disease of swine, shows that this disease is rare in swine. The transmission experiments reported by von Ratz (1913) were made by the use of muscle pulp, a method open to serious objection, as anaerobes other than *C. chauvei* can usually be found in blackleg muscle, especially from field cases. The material used by von Ratz was pathogenic for rabbits, which would suggest that this material was not pure blackleg.

Blackleg has been reported in sheep, the organism having been isolated in Montana during recent years. Marsh (1919, 1920) states that the disease was controlled by the use of blackleg aggressin. Goss et al. (1921) showed that sheep were susceptible to blackleg inoculations, but required rather large doses. He suggests that natural infection is rare.

Cattle are very susceptible to blackleg. Before vaccination was practiced from 10 to 20 per cent of all young stock in the

southwest died of blackleg (Norgaard, 1898). Cattle have been repeatedly shown to be susceptible to infection by the use of pure cultures of *C. chauvei*. Most of the strains used at the Kansas Station have been tested for pathogenicity on cattle. In the production of blackleg agressin, twenty-one of the strains have been used in doses of 5 to 30 mils culture virus to kill calves for the production of agressin. Cultures 1, 2, 3, 4, 5, 6, 9, 10, 12, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 27, 28, 29 and 33 have all produced typical blackleg in cattle. In a number of these cases *C. chauvei* has been reisolated (strains 23 and 27).

Guinea-pigs are quite susceptible to blackleg. Their susceptibility has, however, been found to vary considerably. This variation may be seen in the routine tests for potency of powder vaccine. In this test three guinea-pigs are given 3 mgm. vaccine, three are given 5 mgm., three 7.5 mgm., one 15 mgm., one 25 mgm. and one 40 mgm. It is often found that the guinea-pig receiving 25 mgm., one receiving 5 mgm., and one receiving 3 mgm. die and all others on this test show no lesions whatsoever.

In determining the M.L.D. of a blackleg culture from 3 to 6 guinea-pigs are given graduated doses of the culture. The dose taken as the M.L.D. in this work must kill all guinea-pigs given this or larger doses. Some of the guinea-pigs in such a test receiving less, may, and often do die but these are not considered in determining the M.L.D.

It has been repeatedly found, in attempts to immunize guinea-pigs, and in tests to determine whether there was a relationship between the dose given and the swelling produced, that guinea-pigs receiving $\frac{1}{4}$ or $\frac{1}{5}$ M.L.D. would die while animals getting $\frac{3}{4}$ M.L.D. would live without showing any lesions.

The condition of the animals has considerable effect on their resistance. From 1917 to 1919 all guinea-pigs used at the Kansas Station were purchased from commercial concerns; since 1919 they have been obtained from the Animal Husbandry Department of the Kansas Agricultural College. Up to 1922 they were fed the ordinary ration of oats and bran. Since that time a mixture of alfalfa meal, tankage; bone ash, and mineral salts has been added. The health of the guinea-pigs has been greatly

improved and their resistance to blackleg has been increased. Previous to 1922 the average M.L.D. calculated from 52 tests on approximately 200 guinea-pigs was 0.3 mil. In the period from 1922 to 1924 the M.L.D. calculated from 21 tests on 150 pigs was 0.42 mil or 50 per cent greater. The calf pathogenicity of the cultures was practically the same, 10 to 20 mils culture being sufficient to kill healthy calves during both periods, thus showing that there was an increase in resistance in the guinea-pigs and not a decrease in virulence in the cultures used.

An experiment was started in 1919 in coöperation with Prof. H. L. Ibsen of the Department of Animal Husbandry by which it was hoped to find a race of guinea-pigs of more uniform susceptibility. Animals which had survived inoculations with *C. chauvei* cultures were mated and their offspring tested for blackleg resistance, approximately the same dosage of virus being used as had been used on their parents. This experiment was carried on until 1923 at which time 137 guinea-pigs had been reared and tested. No marked difference in resistance to blackleg could be demonstrated.

Rabbits have proved to be immune to all the strains of *C. chauvei* that have been used. Cultures have been injected both intravenously and subcutaneously in doses of 0.5 to 1.5 mil.

White rats injected with 0.1 mil of cultures 3 and 12 showed no symptoms or lameness. This dose killed guinea-pigs.

White mice have been reported as being susceptible to blackleg, and recently these animals have been used in the determination of the toxicity of the Foth and Kitt types of the organism (Kojima, 1923).

SEROLOGICAL REACTIONS

The macroscopic agglutination test has been used to study the relationship between some of the strains of *C. chauvei* and *C. edematis*. An attempt was also made to classify the three following cultures of *C. chauvei* which showed atypical cultural reactions: Strain "Reddish," obtained from Prof. G. F. Reddish of the University of Virginia, strain "Berg," from the Berg Biological Company, and strain "Lister" from the Lister Institute,

London. All these strains grow quite readily on 2 per cent glucose infusion agar. Slight morphological differences were noted, especially as to the number of organisms, pair and spore formation. In this they differed from typical strains of *C. chauvei*.

Rabbits were immunized to strains 1 and 33 of *C. chauvei*, strains 1, 3, 4 and 5 of *C. edematis* and strains "Reddish," "Berg," and "Lister." The rabbits were given from three to five injections of twenty-four to forty-eight hour brain-liver cultures intravenously or subcutaneously. Strains C.E. 2 and C.E. 5 produced a toxin that killed the first rabbits given these cultures. This toxin was destroyed in later experiments by heating the cultures to 65°C. for ninety minutes. The rabbits immunized against C.E. 2 died of other causes before immunity was developed. One serial of anti-blackleg serum 46 was used. This is a serum produced from horses immunized against strains 1 to 10.

Five dilutions of serum were made in one mil saline. The antigen in the form of twenty-four hour brain-liver cultures was added in doses of 1 mil to each tube. Duplicates of each dilution were made and a control containing no serum was used to assist in the reading of the test and to prevent the reading of auto-agglutination as a positive reaction. The reaction was read after two to six hours incubation and again after ten hours at room temperature. The final dilutions of serum were 1:40, 1:80, 1:240, 1:480 and 1:960.

Table 3 shows the agglutinations obtained.

From these data it is seen that the *C. chauvei* sera agglutinated *C. chauvei* strains in a fairly high dilution. *C. edematis* strains were agglutinated in dilutions of 1:80 at the most. The three atypical strains were agglutinated in dilutions of from 1:40 to 1:240. In one case no agglutination was produced, thus indicating that these strains are not identical with the type used as standard *C. chauvei*. The agglutinations by the *C. edematis* sera show that strain 5 is a type 1 culture, and also that the three atypical *C. chauvei* strains are not the C.E. 1 or C.E. 3 type cultures. If there are only three serological types of *C. edematis* it might be possible to identify C.E. 4 as a type 2 culture. Also

the three atypical *C. chauvei* are not *C. edematis* types. The cultural reactions of strain "Lister" which produced acid in all carbohydrates, using serum water as a base, indicate that it was closely related to *C. edematis* culturally. Serologically it is seen that these three strains are neither *C. edematis* nor *C. chauvei* strains of the type described in this paper.

TOXINS

The production of toxins by *C. chauvei* has been reported by numerous authors since Roux in 1888 reported on the immuniza-

TABLE 3

Agglutination reactions of C. chauvei and C. edematis strains

ANTIGENS	HORSE SERUM 46	RABBIT ANTI-SERA								
		1	33	C.E. 1	C.E. 3	C.E. 4	C.E. 5	CHAUVEI LISTER	CHAUVEI REDDISH	CHAUVEI BERG
1	240	*480*	80	0	0	0	0	40	0	0
10	240	480	960					80	0	0
24	240	480	960	0	0	40	0	80		
33	240	480	*960*	0	0	40	0	80	40	40
C.E. 1	40	40	40	*480*	80	40	480	240	40	0
C.E. 2	40	40	80	40	40	80	80	80	40	80
C.E. 3	0	40	80	40	*480*	80	0	80	40	240
C.E. 4	0	40	80	40	80	*240*	80	40	80	40
C.E. 5	0	80	80	960	80	80	*960*	80	40	80
Lister	240	240	240	80	40	40	40	*240*	240	40
Reddish	80	40	0	80	80	240	0	240	*480*	240
Berg	80	80	40	40	80	240	80	40	240	*240*

Rabbit anti-serum C.E. 2 agglutinated the strains in the following dilutions. C. chauvei 1, 40; 10, 40; 24, 40; 33, 40; C. edematis 1, 80; 2, *960* 3, 80; 4, 480; Lister 240; Reddish 80; Berg 80.

tion of guinea-pigs by means of filtered broth cultures of *C. chauvei* and tissue exudates from cases of blackleg. Leclainches and Valée (1900, 1923) found that cultures of *C. chauvei* produce toxins that kill guinea-pigs in a few hours. Grassberger and Schattenfroh (1904) and Eisenberg (1907) also found powerful toxins in blackleg cultures. Eisenberg, however, used centrifugation instead of filtration for the production of his toxin.

Kelser (1918) stated that toxic filtrates produced greater immunity in calves than non-toxic filtrates. Eichorn (1918) found that blackleg filtrate is toxic. Haslam and Lumb (1919) showed that the protective powers of filtrate had no relationship to its toxicity.

In the production of blackleg aggressin and blackleg filtrate at the Kansas Experiment Station a routine safety test on guinea-

TABLE 4

The non-toxic nature of blackleg filtrates and aggressins

GUINEA PIG		PRODUCT	METHOD OF INJECTION	DOSE	RESULTS		
Number	Weight				1 day	2 days	3 days
	grams			mils			
305	500	Filtrate	Subcutaneous	15	OK	OK	OK
306	500	Filtrate	Subcutaneous	25	OK	OK	OK
307	400	Aggressin	Subcutaneous	15	OK	OK	OK
308	450	Aggressin	Subcutaneous	23	2X	1X	OK
380	250	Non-phenolized aggressin 1	Subcutaneous	1	OK	OK	OK
381	250	Non-phenolized aggressin 1	Subcutaneous	2	OK	OK	OK
430	300	Non-phenolized aggressin 2	Subcutaneous	3	OK	OK	OK
431	300	Non-phenolized aggressin 2	Subcutaneous	6	OK	OK	OK
490	400	1 day filtrate	Subcutaneous	5	2X	OK	OK
491	375	2 day filtrate	Subcutaneous	5	OK	OK	OK
492	300	3 day filtrate	Subcutaneous	5	1X	OK	OK
493	400	6 day filtrate	Subcutaneous	5	OK	OK	OK
494	400	7 day filtrate	Subcutaneous	5	OK	OK	OK
533	400	10 day filtrate	Intracardially	1	OK	OK	OK
537	300	10 day filtrate	Intracardially	1	OK	OK	OK
538	300	10 day filtrate	Intravenously	0.5	OK	OK	OK

1X, slight swelling; 2X, moderate swelling; 3X, large swelling.

pigs is made. Two guinea-pigs are given 7 mils of the product subcutaneously. In all cases where this test has been made on material shown to be sterile by cultural tests on brain liver medium the guinea-pigs have shown practically no swellings. In a few cases where the cultural test and the guinea-pig test were started simultaneously the guinea-pigs have died. In all

these cases the material was shown to be contaminated and in several cases *C. chauvei* has been reisolated from these guinea-pigs. Large doses, 15 to 25 mils of filtrate and aggressin, have been injected into guinea-pigs without producing any lesions, as shown in table 4.

A series of tests on unphenolized filtrates and aggressins was also made; no lesions were produced by these products. A series of flasks were then inoculated with *C. chauvei* 33. After twenty-four hours incubation, one flask was taken, the fluid poured off and filtered through small Mandler filter candles. This was repeated on the second, third, sixth and seventh days. In the case of the first filtrate a guinea-pig was given 5 mils filtrate, and a culture tube containing brain liver medium was inoculated with the same amount (5 mils). The guinea-pig was dead the next morning and the culture tube showed cloudiness and gas. Both were shown to contain *C. chauvei*. This filtrate was refiltered and together with the other daily filtrates was tested for sterility before injecting other guinea-pig. Table 4 shows that these filtrates produced no lesions in guinea-pigs, except in the case of filtrates 1 and 3 which were contaminated by a few organisms; this was shown by growth of the brain liver test after three days incubation. It was found that sterile filtrates were much more easily obtained from old cultures that had auto-agglutinated than from cultures containing vegetative cells. Non-phenolized filtrates of fresh exudates and muscle juices obtained from calves that had died of spontaneous blackleg were also shown to be non-toxic (table 4). Intravenous and intracardiac injections of filtrate were also shown to be non-toxic.

AGGRESSINS

The substances found in filtered muscle juices and exudates of blackleg lesions (aggressins), or in filtered cultures of *C. chauvei* (filtrates) have been shown to be non-toxic (table 4). These products are shown to be true aggressins by the fact that (1) the addition of small amounts of these products will activate non-lethal doses of blackleg virus, (2) the addition of small amounts of these products to small amounts of avirulent washed cultures

of *C. chauvei* will produce typical blackleg lesions in guinea-pigs, (3) the addition of small amounts of these products to *C. chauvei* cultures will produce death in guinea-pigs passively immunized against blackleg.

Table 5 shows that when a small amount of aggressin was added to 1/10 M.L.D. (0.07 mil) blackleg culture, death of the guinea-pig was produced in two days, and when 1 mil filtrate was added to 1/5 M.L.D. death was produced in four days. These experiments show that sublethal doses of blackleg culture virus are activated by small amounts of the products of growth of *C. chauvei*, either in the calf as in the case of the aggressin, or in culture media as in the case of filtrate.

TABLE 5

The aggressiveness of blackleg aggressin and filtrate when added to $\frac{1}{10}$ and $\frac{1}{5}$ M.L.D. C. chauvei culture

GUINEA-PIG		PRODUCT	DOSE	CULTURE	DOSE M.L.D.	RESULTS			
Number	Weight					1 day	2 days	3 days	4 days
			mil						
389	275	Aggressin	0.75	33	1/10	OK	D		
710	300	Filtrate	1.0	33	1/5	OK	2X	3X	D

2X, moderate swelling; 3X, large swelling; D, death.

The same reaction has been shown to occur in cattle (Scott, 1923). Several cases of death from blackleg in calves treated with blackleg filtrate have been reported to the Kansas Agricultural College. It was found that these calves had been vaccinated with blackleg powder vaccine some weeks before. The disease in every case developed a few days after the use of the filtrate in the region of the neck where the vaccine had been injected, thus showing the aggressiveness of this product.

If cultures of *C. chauvei* are washed by centrifugation in salt solution so as to free the cells from all the products of growth an avirulent product remains (table 6). If small amounts of this washed culture be added to small amounts of filtrate or aggressin a virulent product results. Table 6 shows that by washing cultures of *C. chauvei* three times in salt solution and diluting the resulting packed cells in salt solution up to the original volume

an absolutely avirulent product results. The large doses may produce slight swellings due to the large amounts of cells to be absorbed; these swellings are more in the nature of absorption swellings than inflammatory swellings. The M.L.D. of culture 28 was 0.2 mil before washing and after centrifugation three times in salt solution a dose of 15 mils or the equivalent of 75 times the M.L.D. is see to be avirulent, this amount only producing a slight absorbtion swelling in guinea-pig 441.

TABLE 6

Avirulence of washed culture

GUINEA-PIG		WASHED CULTURE	DOSE	RESULTS			
Number	Weight			1 day	2 days	3 days	4 days
			mils				
441	600	28	15	1X	2X	OK	OK
442	600	28	7	OK	OK	OK	OK
330	500	3	10	OK	OK	OK	OK
830	700	2	15	1X	1X	OK	OK

1X, slight swelling; 2X, moderate swelling.

TABLE 7

Aggressiveness of aggressin and filtrate in the presence of washed culture

GUINEA-PIG		WASHED CULTURE		PRODUCT	DOSE	RESULTS		
Number	Weight	Number	Dose			1 day	2 days	3 days
			mil		*mil*			
119	300	28	0.3	Aggressin	0.3	D		
126	350	28	0.3	Filtrate	0.3	3X	D	

3X, large swelling; D, death.

It is therefore seen that the addition of the products of growth of *C. chauvei* will reactivate the washed culture.

When 0.03 mil of washed culture 28 shown to be avirulent in doses of 15 mils (table 6) was added to blackleg aggressin or filtrate and guinea-pigs were injected with these mixtures they died of typical blackleg as shown in table 7. This demonstrates that the products of growth of *C. chauvei* contain an aggressive substance.

If guinea-pigs are passively immunized by the injection of anti-

blackleg serum and fifteen hours later a mixture of *C. chauvei* culture and filtrate or aggressin is injected, an aggressive reaction will be produced resulting in the death of the guinea pig from blackleg as shown in table 8. This reaction has been called the Neutralization reaction.

Table 8 shows that amounts of anti-blackleg serum sufficient to protect a guinea-pig against at least 15 M.L.D. of *C. chauvei* culture virus, were neutralized by the injection of a mixture of 0.3 mil blackleg filtrate and aggressin with 1.5 M.L.D. *C. chauvei* culture fifteen hours later. This shows that these products contain an aggressive substance. Guinea-pig 684, given anti-blackleg serum, was protected against 16 M.L.D. of the same culture when given unmixed with aggressin or filtrate.

TABLE 8

Neutralization reaction

GUINEA-PIG		SERUM		PRODUCT	DOSE	DATE	VIRUS	D 48 M.L.D.	DATE	RESULTS		
Number	Weight	Dose	Date							1 day	2 days	3 days
		mil			*mil*							
394	250	0.15	12/5	Aggressin	3	12/6	28	1.5	12/6	3X	3X	D
398	250	0.15		Filtrate	3		28	1.5		3X	3X	D
684	200	0.15					28	16.0		½X	OK	OK

3X, large swelling; D, Death.

The neutralization reaction shown in table 8 forms the basis for the neutralization potency test for blackleg filtrate and aggressin (Goss and Scott, 1918). In the neutralization test a series of guinea-pigs are given a known amount of anti-blackleg serum, the usual dose being 15 anti-blackleg units (one anti-blackleg unit of serum is that amount which will protect a guinea-pig against one M.L.D. of blackleg virus). Fifteen hours later these guinea-pigs are given from 1 to 3 M.L.D. blackleg culture virus mixed with graduated doses (1, 2, 3, 4 and 5 mils) of blackleg filtrate or aggressin. Some of these guinea-pigs will die or show marked lesions. The guinea-pig dying from the smallest dose of filtrate or aggressin determines the strength of the prod-

uct. This amount will contain 15 minus 2 (dose of serum minus dose of virus), or 13 aggressive units from which the aggressive units in a 5 mil dose can be calculated.

The reaction in table 7 showing the activation of the avirulent washed culture is made use of in the washed culture potency test for blackleg filtrate and aggressin (Scott, 1923). In the washed culture test the smallest amount of washed culture that is activated by small amounts of filtrate or aggressin (0.3 to 0.7 mil) is taken to be the unit of washed culture. This unit is called the "potential" M.L.D. In determining the aggressive strength of filtrate or aggressin by means of the washed culture test a series of guinea-pigs are given one potential M.L.D. of washed culture mixed with graduated doses (0.15, 0.25, 0.3 and 0.4 mil) of filtrate or aggressin. The smallest amount of these products that activates the washed culture determines the strength of that product. This amount is called one aggressive unit.

In comparing filtrates and aggressins by means of these two tests five important points have been brought out.

1. The aggressive strength of various serials of filtrate and aggressin, as tested by these methods, was fairly constant and the strengths, as determined by several repetitions of the same test, were also very close. In table 9 a comparison of several such serials has been made. Serials numbered, strain 1 to 32, are filtrates made from stock cultures of *C. chauvei* having these numbers.

Table 9 shows that there is not much difference in the amount of aggressive substance produced by different strains of *C. chauvei*. It also shows that the neutralization and washed culture tests give very nearly the same readings for the different products tested and that successive tests do not vary to any marked extent. The average of 47 neutralization tests gives a reading of 16.35 aggressive units and the 38 washed culture tests on the same 27 products a reading of 15.53 aggressive units. Filtrate "28" was made from a virulent culture of this organism having an M.L.D. of 0.2 mil and filtrate "avirulent 28" was made from a culture of this strain having an M.L.D. of 0.9 mil. In both cases the M.L.D. was determined from the culture before filtration. It is

seen that the filtrates from both the virulent and avirulent cultures have an aggressive strength of 16.5 aggressive units.

2. The aggressiveness of filtrates made from cultures of virulent and avirulent strains shows no marked differences, which indicates

TABLE 9

Comparison of aggressiveness of filtrates and aggressins

	NEUTRALIZATION TEST				WASHED CULTURE TEST		
	1st test	2nd test	3rd test	4th test	1st test	2nd test	3rd test
Filtrate 1195	21.5				16.65		
Filtrate 1196	13.0	16.5			12.5		
Filtrate 197	13.0	16.5			12.5	12.5	
Filtrate 298	13.0				12.5	16.5	
Filtrate 299	10.0	16.5			10.0		
Filtrate 310	16.5	16.5			20.0		
Filtrate 101	13.0	16.5			20.0		
Filtrate 102	16.5	16.5			14.28	16.55	
Strain 1	13.0	13.0			14.65	16.65	
Strain 2	13.0				12.5		
Strain 3	13.0				12.5		
Strain 5	16.5				10.0		
Strain 6	21.5				12.5		
Strain 10	13.0	16.5	16.5		16.65	16.65	16.65
Strain 12	16.5	16.5			14.28		
Strain 19	16.5	16.5			33.33		
Strain 23	16.5	21.65			14.28		
Strain 24	21.65	21.65			16.65		
Strain 27	32.5				16.65	16.65	16.65
Strain 28	13.0	16.5	16.5		16.65	14.28	
Strain 29	13.0				9.0		
Strain 30	13.0				12.5		
Strain 31	16.5	16.5			16.65		
Strain 32	16.5				16.65		
Avirulent 28	13.0	16.5	16.5	16.5	16.65	16.65	14.28
Aggressin 207	13.0				16.65		
Aggressin 208	21.5	21.5			16.65		
	47 tests Average, 16.35				38 tests Average, 15.53		

that the lethal part of a virulent culture is in the cells and not in the products of growth.

Table 10 shows the aggressiveness of filtrates prepared from

cultures having different M.L.D. values. Each culture was first tested to determine its M.L.D. and was then filtered. Strain 28 had an M.L.D. of 0.2 in one case and 0.9 in the other. It

TABLE 10

Comparison of aggressiveness of virulent and avirulent strains of C. chauvei

AVIRULENT STRAINS			VIRULENT STRAINS		
Number	M.L.D.	Aggressive strength (A. U.)	Number	M.L.D.	Aggressive strength (A. U.)
	mil			*mil*	
1	>1	14.5	28	0.2	16.5
2	>1	13.0	29	0.3	13.0
12	>1	16.5	32	0.2	16.5
28	0.9	16.5			

TABLE 11

The action of heat on aggressive substances

PRODUCT	PRODUCTION TESTS		UNHEATED TESTS		HEATED AT 60°C.					
					15 minutes		30 minutes		60 minutes	
	Neutralization A. U.	Washed culture A. U.	Neutralization A. U.	Washed culture A. U.	Neutralization A. U.	Washed culture A. U.	Neutralization A. U.	Washed culture A. U.	Neutralization A. U.	Washed culture A. U.
	1922		*1923*							
Fil. 102	16.5	14.28	16.5	16.65	21.65	33.33	21.65	—	32.5	32.33

PRODUCT	PRODUCTION TESTS 1920–1922		UNHEATED TESTS 1923			HEATED AT 60° FOR 60 MINUTES	
	Neutralization A. U.		Neutralization A. U.		Washed culture A. U.	Neutralization A. U.	Washed culture A. U.
Agg. 205	21.65		21.65	16.5	20.0	32.5	33.33
Agg. 206	21.65		21.65	21.65	20.0	32.5	33.33
Agg. 209	21.65		21.65	21.65	20.0	32.5	33.33
Fil. 1195	21.65		21.65		20.0	32.5	27.0
Fil. 1196	13.0	16.5	13.0		20.0	24.5	33.33
Fil. 298	12.5	16.5	21.65		16.5	16.5	22.0

is seen that the virulence of the cultures does not materially affect the aggressive strength of the filtrate produced. It is also seen that one strain does not have a constant M.L.D.

3. The potential M.L.D. of the washed culture was found to be proportional to the M.L.D. of the unwashed culture. It was found to be from 0.05 to 0.2 mil greater than the corresponding M.L.D. The same amount of agressive substance was required to activate the potential M.L.D. of cultures having a high virulence as for those having a low virulence. Washed cultures made from avirulent strains may have a potential M.L.D. but this could not be determined. The cells of these avirulent cultures therefore contain little or no lethal substance but as has been seen (table 10) they produce as much aggressive substance as the virulent strains.

4. The action of heat on the aggressive substances increases their activity. This point is referred to in Karsner and Ecker's text, "The Principles of Immunology," who cite Bail's work as authority.

TABLE 12

Hydrogen-ion concentration of filtrate before and after the application of heat

FILTRATE	pH BEFORE HEATING	pH AFTER HEATING 60° FOR ONE HOUR
280	6.6	6.5
101	5.5	5.6
108	6.1	6.2
1195	5.4	5.5

Table 11 shows that heating blackleg filtrates or aggressins at 60°C. for one hour increases the aggressiveness of these products.

This action is not due to changes in the hydrogen-ion concentration, as is shown by table 12 which gives the pH reading before and after heating four serials of blackleg filtrate. One, filtrate 1195, was the same as that tested in table 11.

5. The keeping qualities of blackleg filtrate and aggressin were found to be high. Three aggressins produced in 1920 having an aggressive strength of 21.5, 13.0, and 16.25 aggressive units had in 1923 an aggressive strength of 13.0, 16.25, and 21.65 aggressive units respectively. Filtrate 1, having an aggressive strength of 13.5 in 1918 had an aggressive strength of 9 in 1923, and after being held in the incubator for forty weeks had a strength of 26.0 aggressive units.

SPECIFICITY OF AGGRESSIVE SUBSTANCES

The aggressive substances found in filtrates made from cultures of *C. chauvei* and *C. edematis* are essentially non-specific as far as can be determined by guinea pig tests. In some cases where the dose of virus and of aggressive substance and the resistance of the guinea-pig are favorable, specificity can be demonstrated as shown in table 13.

TABLE 13

Specificity reactions of aggressive substance

GUINEA-PIG		VIRUS	DOSE M.L.D.	FILTRATE	DOSE	RESULTS		
Number	Weight					1 day	2 days	3 days
					ml			
549	325	C.E. 1	1/10	C.E 1	0.75	OK	OK	OK
550	350	C.E. 1	1/10	C.E. 2	0.75	OK	OK	OK
551	375	C.E. 1	1/10	C.E. 3	0.75	OK	OK	OK
552	300	C.E. 1	1/10	Ch. 33	0.75	OK	OK	OK
553	325	C.E. 2	1/10	C.E. 1	0.75	OK	OK	OK
554	375	C.E. 2	1/10	C.E. 2	0.75	OK	OK	D
555	350	C.E. 2	1/10	C.E. 3	0.75	OK	OK	OK
556	325	C.E. 2	1/10	Ch. 33	0.75	OK	OK	OK
634	250	C.E. 3	1/5	C.E. 1	0.75	OK	OK	OK
635	225	C.E. 3	1/5	C.E. 2	0.75	OK	OK	OK
636	300	C.E. 3	1/5	C.E. 3	0.75	1X	1X	1X
637	350	C.E. 3	1/5	Ch. 33	0.75	OK	OK	OK
671	325	Ch. 33	1/4	C.E. 1	1.25	3X	D	
672	275	Ch. 33	1/4	C.E. 2	1.25	3X	D	
673	275	Ch. 33	1/4	C.E. 3	1.25	3X	D	
670	300	Ch. 33	1/4	Ch. 33	1.25	1X	OK	OK

The nonspecific reaction is also seen in the fact that filtrates of *C. edematis* strains will activate *C. chauvei* virus in the neutralization test.

Table 13 shows that in the use of *C. edematis* Type 1 culture no aggressiveness was produced by any filtrates. In the case of *C. edematis* Type 2 culture true specificity was shown by filtrate *C. edematis* 2 which was the only filtrate to produce a reaction in the guinea-pigs C.E. 2 culture. The test on culture *C. edematis* 3 also shows specificity but the test using *C. chauvei* 33 shows a non specific reaction. The filtrates of *C. edematis*

1, 2 and 3 contained enough aggressive substance to activate the culture virus of *C. chauvei*, while the filtrate of *C. chauvei* 33 did not contain sufficient aggressive substance to produce more than a very slight reaction in any case.

The finding that the aggressive substance in blackleg aggressins and filtrate is non-specific when tested on guinea-pigs and the fact that filtrates and aggressins produced from typical strains of *C. chauvei* produce a lasting immunity in cattle but not in guinea pigs, while filtrates produced from atypical strains or from other organisms do not produce these results, leads to the supposition that the aggressive substances may not be the immunizing agents.

IMMUNITY

Immunization of cattle against blackleg has been carried on since Arloing Cornevin and Thomas (1887) introduced the powdered muscle tissue or Lyons backleg vaccine. Leclainchee and Valée (1913) introduced the liquid spore vaccine which is produced by growing cultures of *C. chauvei* at temperatures of 42°C. In 1916 Franklin and Haslam (Goss, 1917), working at the Kansas State Agricultural College introduced blackleg aggressin, which is produced by filtering the muscle juices and exudates obtained from a case of blackleg. In 1917 Goss and Scott (Goss, 1919) developed the artificial aggressin, or culture filtrate, produced by filtering pure cultures of *C. chauvei*.

The results from the use of powder vaccine are not satisfactory. The immunity produced is of rather short duration and very susceptible calves may be given blackleg. The liquid spore vaccine has proved to be very satisfactory. Blackleg filtrate and aggressin produce a high degree of immunity (Scott, 1923) which protects the animals during their period of greatest susceptibility (from six months to two years of age), if these products are given at the age of five or six months of age. Vaccination with powder vaccines reduced the losses from 10 to 20 per cent to 1 per cent (Norgaard 1898). Vaccination with blackleg filtrate and aggressin have reduced the losses to 1 in 10,000 or less.

Immunization of guinea-pigs by means of blackleg muscle virus, *C. chauvei* cultures, serum virus mixtures and filtrates and aggressins does not give uniform results. Immunization with cultures and muscle virus may perhaps give slightly greater immunity than when filtrates or aggressins are used. Of 138 guinea-pigs injected with from 2 to 7 mils blackleg aggressin and ten days to two weeks later with 1 to 2 M.L.D. *C. chauvei* culture, 70, or 50.7 per cent died. Of 270 guinea-pigs injected with 2 to 7 mil blackleg, filtrate and ten days to two weeks later with 1 to 2 M.L.D. *C. chauvei* culture, or muscle virus, 128, or 47.4 per cent died. These experiments show that the immunity produced by the use of these products is very low. This immunity does not always exceed the limits of natural variation in resistance of guinea-pigs. It is possible that all the pigs that died were animals which had a very low initial resistance and that their resistance was increased by the injection of these products but not to a degree sufficient to protect them against the test dose.

The cross immunization tests showing immunization of guinea-pigs by whole cultures of *C. chauvei* and *C. edematis* (table 14a) show that strain *C. chauvei* 33 immunized guinea-pigs against itself but not against *C. edematis* strains 1, 2, or 3. The *C. edematis* strains protected guinea pigs against themselves and also showed some protection against strains not used in the immunization. Immunization by the use of culture filtrates (table 14b) shows irregular protection.

In these experiments with culture immunization it is seen that guinea-pigs immunized by the injection of culture *C. chauvei* 33 were only protected against this culture but not against *C. edematis* strains 1, 2 or 3. *C. edematis* 1 protected a guinea pig against itself and against *C. edematis* 2. *C. edematis* 2 protected against *C. edematis* 1 but not against itself. *C. edematis* 3 protected guinea-pigs against itself and against *C. chauvei* 33. In the series of tests using filtrates of these cultures it is seen that filtrate of *C. chauvei* culture 33 protected against *C. edematis* 1 and 3 but not against itself. *C. edematis* 1 filtrate protected guinea-pigs against the culture itself but not against the other

TABLE 14

Cross immunization tests

GUINEA-PIG		IMMUNIZATION CULTURE	DOSE	DATE	TEST CULTURE	DOSE M.L.D.	DATE	RESULTS		
Number	Weight							1 day	2 days	3 days
					(a)					
			mil							
354	325	Ch. 33	0.35	1/18	Ch. 33	1.3	2/2	OK	OK	OK
355	300	Ch. 33	0.35	1/18	C.E. 1	1.3	2/2	3X	D	
356	300	Ch. 33	0.35		C.E. 2	1.3		D		
357	300	Ch. 33	0.35		C. E. 3	1.3		D		
350	250	C.E. 1	0.1		Ch. 33	1.3		3X	D	
349	300	C.E. 1	0.1		C.E. 1	1.3		OK	OK	OK
375	250	C.E. 1	0.025	1/20	C.E. 2	1.3		OK	OK	OK
376	250	C.E. 1	0.025		C.E. 3	1.3		D		
361	300	C.E. 3	0.1	1/18	Ch. 33	1.3		2X	1X	OK
363	300	C.E. 3	0.1		C.E. 1	1.3		3X	D	
378	250	C.E. 3	0.025	1/20	C.E. 2	1.3		OK	D	
379	250	C.E. 3	0.025		C.E. 3	1.3		OK	OK	OK
364	300	C.E. 2	0.025		C.E. 1	1.3		2X	1X	OK
366	250	C.E. 2	0.025		C.E. 2	1.3		OK	OK	D
382	250	C.E. 2	0.01	1/23	C.E. 3	1.3		D		
					(b)					
		FILTRATE								
601	300	F.C.E. 1	4	3/29	C.E. 1	1	4/7	1X	OK	OK
602	275	F.C.E. 1	4		C.E. 2	1		D		
603	300	F.C.E. 1	4		C.E. 3	1		D		
604	275	F.C.E. 1	4		Ch. 33	1		3X	D	
605	275	F.C.E. 2	4		C.E. 1	1		2X	D	
606	275	F.C.E. 2	4		C.E. 2	1		D		
607	300	F.C.E. 2	4		C.E. 3	1		D		
608	300	F.C.E. 2	4		Ch. 33	1		3X	D	
609	225	F.C.E. 3	4		C.E. 1	1		OK	OK	OK
610	300	F.C.E. 3	4		C.E. 2	1		D		
547	375	F.C.E. 3	5		C.E. 3	1		D		
612	300	F.C.E. 3	4		Ch.33	1		3X	D	
613	225	F.Ch. 33	4		C.E. 1	1		OK	OK	OK
614	250	F.Ch. 33	4		C.E. 2	1		D		
615	250	F.Ch. 33	4		C.E. 3	1		OK	OK	OK
616	250	F.Ch. 33	4		Ch. 33	1		D		

strains. *C. edematis* 2 filtrate did not protect guinea pigs against any cultures used. *C. edematis* 3 filtrate protected only against *C. edematis* 1 culture.

Similar results were obtained in a series of tests on the immunization of guinea pigs by means of filtrates injected in doses of 5 to 10 mils given in six injections over a period of seven days.

From these experiments it is seen that immunization of guinea-pigs by means of whole cultures does not give satisfactory results, and that immunization of guinea-pigs by means of

TABLE 15

Passive immunization of guinea-pigs

GUINEA-PIG		SERUM	DOSE	DATE	CULTURE	DOSE M.L.D.	DATE	RESULTS		
Number	WEIGHT							1 day	2 days	3 days
			mil							
251	200	46	0.07	11/28	Ch. 33	5	11/29	OK	OK	OK
252	200	46	0.07		C.E. 4	5	11/29	D		
253	150	46	0.07		C.E. 1	5		D		
254	250	46	0.07		C.E. 2	5		D		
708	350	46	0.05	5/5	C.E. 3	3	5/6	D		
709	250	46	0.05		C.E. 5	3		D		
703	300	C.E. 5	1.25	5/5	C.E. 1	1.5	5/6	OK	OK	OK
704	250	C.E. 5	1.25		C.E. 2	1.5		D		
705	350	C.E. 5	1.25		C.E. 3	1.5		D		
706	350	C.E. 5	1.25		C.E. 4	1.5		D		
711	300	C.E. 5	1.25		C.E. 5	1.5		2X	3X	D
712	300	C.E. 5	1.25		Ch. 33	1.5		D		

2X, slight swelling; 3X, large swelling; D, death.

filtrates is even less satisfactory. In neither case is the immunization of sufficient regularity to be used as a basis for differentiation of strains.

Immunization of guinea-pigs by the use of anti-sera on the other hand, gives uniform results, if sufficient serum is given to protect the guinea pigs against at least 2 M.L.D. of culture. In this way the variation in susceptibility of the guinea-pigs is minimized. Table 15 shows a series of passive immunization tests.

Anti-blackleg serum 46 was produced from horses immunized

against strains 1 to 10. The anti-*C. edematis* serum was a rabbit serum produced by the injection of whole cultures of *C. edematis* 5 intravenously and subcutaneously. Serum 46 protected guinea pigs against *C. chauvei* strain 33 but not against any of the *C. edematis* strains. Serum 46 has also been used to immunize guinea-pigs against most of the other strains of *C. chauvei* and has protected guinea-pigs against these strains in every case. The serum produced from a rabbit immunized against *C. edematis* 5 was rather weak, protecting guinea-pigs only against 1.5 M.L.D. (1.25 mil). Guinea-pig 711 was immunized against C.E. 5 by the use of anti-serum. This guinea-pig died on the third day following the test dose of C.E. 5. This shows the importance of having enough latitude between the expected protective strength of the serum to be used and the test dose of the virus. Guinea-pig 703 immunized against C.E. 5 and tested with *C. edematis* strain 1 showed no swellings. This shows that C.E. 5 is a type 1 strain and confirms the results found in the agglutination reaction (table 3) which also showed that *C. edematis* 5 was a type 1 strain.

SUMMARY

1. It has been shown that anti-blackleg serum from horses protected guinea-pigs against *C. chauvei* strains isolated from typical cases of blackleg disease. These strains did not grow in 2 per cent glucose infusion agar.

2. *C. chauvei* strains were shown to be typically single Gram-positive rods, producing occasional oval subterminal spores. *C. edematis* strains were seen to be typically chain forming types showing numerous filamentous rods. Some strains of *C. edematis* produced a predominance of paired organisms, in a number of which the elements formed a sharp angle one with another.

3. *C. chauvei* strains were shown to be much less active biochemically than the *C. edematis* strains, *C. chauvei* only fermenting carbohydrates in the presence of unheated serum, while the *C. edematis* strains fermented carbohydrates in a greater number of media. *C. chauvei* ferments a restricted number of

carbohydrates only while the *C. edematis* types ferment most carbohydrates.

4. The pathogenicity of the cultures of *C. chauvei* for guinea-pigs was not constant. This was partly due to dfferences in resistance of individual guinea-pigs. Changes in culture media apparently did not affect the pathogenicity of *C. chauvei* for guinea-pigs to any marked extent, but the use of a feed rich in vitamines and mineral salts increased the resistance of the guinea-pigs. *C. edematis* type strains were more pathogenic for guinea-pigs than *C. chauvei* strains and were also in some cases highly pathogenic for rabbits and white rats.

5. There was found to be no direct relationship between the pathogenicity of *C. chauvei* cultures for guinea-pigs and their pathogenicity for calves.

6. The agglutination tests showed positive serological differences between the types studied.

7. Both tissue extract and culture filtrates were shown to be non-toxic in doses up to 25 mils.

8. An aggressive substance was demonstrated in both the tissue extract and culture filtrates. This aggressive substance was increased in potency by the action of heat.

9. The absolute specificity of the aggressive substance could not be demonstrated, cross aggressin reactions being apparent in many cases.

10. The amount of aggressive substance produced by a virulent strain was found to be no greater than that produced by an avirulent strain.

11. Active immunization of cattle by means of attenuated cultures, and especially by the use of filtrates and aggressins has been demonstrated, but the active immunization of guinea-pigs against both *C. chauvei* and *C. edematis* was shown to be uncertain.

12. Passive immunization of guinea-pigs by means of antisera proved to be specific and gave a highly reliable method of differentiation.

13. The results obtained by passive immunization reactions confirmed the findings obtained in the agglutination test.

14. Two potency tests based on the production of aggressive substance in cultures of *C. chauvei* and in the tissues of calves affected with blackleg are described.

15. It is shown that the lethal substance is found in the cells of *C. chauvei* and that this lethal substance can not produce death in the absence of products of growth. The amount of this substance present in any given culture determines its virulence.

16. The eight criteria of purity described in connection with the isolation of *C. chauvei* strains were shown to be sufficient evidence of the identity of these strains.

17. The study of three atypical *C. chauvei* strains indicates the possibility of there being two types of *C. chauvei*, such as have been described by Zeiszler and Kojima.

18. The *C. chauvei* type described in this paper appears to be the most prevalent. Anti-sera, filtrates and aggressins produced from this type have been shown to prevent blackleg in all parts of the United States, Mexico, Central America and in South Africa.

REFERENCES

ARLOING, CORNEVIN AND THOMAS 1887 Le charbon symptomatique du boeuf. Ed. 2, Paris.

ASCOLI, A. 1913 Ergebnisse und Ausblicke der Thermopräzipitinreaktion. Virchow's Arch., **213**, 181; Cent. f. Bakt., Abt. 1, Ref., **59**, 675.

BALAVAINE, R. 1909 Die Schützimpfung des Rindes gegen dem Rauschbrand in der Schweiz und in einigem andern Landes. Schweiz. Arch. f. Tierch., **51**, no. 3; Cent. f. Bakt., Abt. 1, Ref., **45**, 629.

BERG, WM. N. 1918 Concentration of symptomatic anthrax (blackleg) toxin. Jour. Agric. Res., **14**, no. 6, 263–264.

BOHLER 1917 Gasbrand beim Pferde verursacht durch rauschbrandähnliche Bazillen. Ebenda, 1917, p. 181; Cent. f. Bakt., Abt. 1, Ref., **67**, 72.

BUSHNELL, L. D. 1922 A method for the cultivation of anaerobes. Jour. Bact., **7**, 277–283.

CONRADI, H., UND BEILING, R. 1916 Zur Oetiologie und Pathogenese des Gasbrandes. Münch. Med. Woch., **43**; Bul. Pasteur, **14**, 467.

CHRISTIANSEN, M. 1920 Der Walfischseptikämiebazillus und sein verhältnis zur Oedembazillengruppe. Cent. f. Bakt., Abt. 1, Orig., **84**, 127–129.

DETRE, D. 1913 Über das Rauschbrandserum. Virchow's Arch., **213**, 284; Cent. f. Bakt., Abt. 1, Ref., **59**, 698.

DIEDERICHS, F. 1911 Zur Rauschbrandfrage bein Pferde. Deutsch. tierärztl. Woch., **19**, 97–99; Cent. f. Bakt., Abt. 1, Ref., **50**, 455.

EICHORN, A. 1917 Blackleg Filtrate. Jour. Amer. Vet. Med. Assoc., **6**, no. 4, p. 406.

EICHORN, A. 1918 Studies in blackleg immunization with special reference to blackleg filtrate. Jour. Amer. Vet. Med. Assoc., **5**, no. 6, 653–659.

EISENBERG, PH. 1917 Sur la toxine du bacille du charbon symptomatique. C. R. Soc. Biol., **42**, 613; Bulletin Pasteur, **5**, 488.

FOTH, H. 1909 Die Diagnose des Rauschbrandes. Zeitschr. f. Infektionskrank. der Haust., **6**, 201.

FOTH, H. 1910 Die bakteriologische diagnose des Milzbrandes und des Rauschbrandes in Veterinarpolizeilichen Praxis. Arch. f. Wiss. u. Prakt. Teirheilk, p. 93–107; Cont. f. Bakt., Abt. 1, Ref., **48**, 243–244.

FOTH, H. 1910 Die Diagnose des Rauschbrandes. Zeischr. f. Inf. Paras. Krank v. Hyg. d. Haust., no. 8, 117–139; Cent. f. Bakt., Abt. 1, Ref., **49**, 180.

FOTH, H. 1918 The valuation of the new symptomatic anthrax vaccine (Emphysarcol) Berl. tierarztl. Woch. **34**, 171–175; Veterinary Review **4**, 433.

FRANKLIN, O. M., AND HASLAM, T. P. 1916 The strength and composition of blackleg vaccines. Journ. Inf. Dis., **19**, 408–415.

FURTH 1917 Beiträge zur Kenntniss der gasgranderreger. Münch, med. Woch., 1169–1170; Bul. Pasteur, **14**, 771–772.

FROMMELD, J. A. 1917 Die desinfektorische Wirkung des Lichtes auf Mastitis Streptokokken, Rauschbrand, Tetanus und Botulinus. Monatsh. f. prakt. Tierheilk., p. 209; Cent. f. Bakt., Abt. 1, Ref., **67**, 341.

GOCHENOUR, W. S. 1920 Germ-free filtrates as antigens in the complement fixation test. Jour. Agric. Research, **19**, 513–515.

GÖERTTLER, V. 1923 The differentiation of blackleg and similar organisms by a complicated animal test. Zeitschr. Immunit. u. Expt. Therapie. Abt. 1, Orig., **36**, 463–481; Expt. Sta. Rec., **49**, 680.

GOSS, L. W. 1917. Methods of controlling blackleg. Kansas Agric. Expt. Sta. Circ. 59 (revised).

GOSS, L. W. 1919 Blackleg and its control. Kansas Agric. Expt. Sta. Circ. 75.

GOSS, L. W., AND SCOTT, J. P. 1918 Standardization of blackleg vaccines. Jour. Amer. Vet. Med. Assoc., **7**, 24–243.

GOSS, L. W., BARBARIN, R. E., AND HAINES, A. W. 1921 Some characteristics of B. Chauvoei. Jour. Inf. Dis., **29**, 615–629.

GRASSBERGER, R. 1903 Morphologie des Rauschbrandbacillus. Arch. f. Hyg. **48**, Bulletin Pasteur, **1**, 720.

GRASSBERGER, R., AND SCHATTENFROH, A. 1904 Ueber das Rauschbrandgift und ein antitoxisches Serum. Franz Deuticke publishers Leipzig. Bulletin Pasteur, **2**, 316–319.

GRASSBERGER, R., AND SCHALLENFROH, A. 1907 Immunitätsfrage. Wiener klin. Woch. no. 42, Cent. f. Bakt., Abt. 1, Ref., **41**, 575–577.

GRASSBERGER, R., AND SCHATTENFROH, A. 1913 Zur Rauschbranddiagnose. Berl. tierärztl. Woch. no. 50, p. 889; Cent. f. Bakt., Abt. 1, Ref., **61**, 183.

GRAUB, E. AND ZSCHOKKE, W. 1920 Die immunizierung gegen Rauschbrand mit Keim-freien Filtraten. Schweiz. Arch. f. Tierheil., **42**, 52–65, 112–122; Veterinary Review, **4**, 365.

GROSSO, G. 1911 Über die Bedeutung der Agglutination in der Rauschbranddiagnose und über die Gärungsfähigkeit des Rauschbrandbacillus und die diesbezüglichen Unterschiede zwischen Rauschbrand und Malignen Ödem. Berl. tierärztl. Woch., no. 35, 621; Cent. f. Bakt., Abt. 1, Ref., **51**, 402.

HALL, I. C. 1922 Differentiation and identification of the sporulating anaerobes. Jour. Inf. Dis., **30**, 445–504.

HASENKAMP 1909 Rauschbrand bei einem Pferde. Deutsche tierärztl. Woch., p. 471; Cent. f. Bakt., Abt. 1, Ref., **45**, 610.

HASLAM, T. P., AND LUMB, J. W. 1919 Blackleg Toxin. Jour. Inf. Dis., **24**, 362–365.

HASLAM, T. P. 1920 Immunization with blackleg aggressin. Jour. Immun., **5** 539–546.

HASLAM, T. P., AND FRANKLIN, O. M. 1920 Experiments on immunization with pseudo-blackleg pellets. Jour. Inf. Dis., **26**, 424–426.

HECHT, VICTOR 1913 Die Präzipitinreaktion des Rauschbrandes mit einem Beitrage zur frage der Thermoresistenz der Präzipitinogene. Cent. f. Bakt., Abt. 1, Orig., **67**, 371–380.

HEELSBERGEN, T. VAN 1919 Boutvuur maligne oedeem en 'Gasbrand' bacillen. Tijdschr. v. Diergeneesk, **45**, 153–180. Abst. Bact., **5**, 64–65.

HELLER, H. H. 1920 Etiology of acute gangrenous infections of animals. A discussion of blackleg, braxy, malignant edema and whale septicemia. Jour. Inf. Dis., **27**, 385–451.

HIBLER, E. VON. 1908 Untersuchungen über die pathogenen Anaeroben. Jena.

HÖLZEL, E. 1913 Beiträge zur Züchtung, Isolierung und Desinfektion des Rauschbrandbacillus. Cent. f. Bakt., Abt. 1, Orig., **71**, 147.

JENSEN, CO. O. 1915 Die Atiologie der Bradsot. Ztschr. f. d. Haust. 1915, 1–18; Abst. Bact., **7**, 181.

KABSNER, H. T., AND ECKER, E. E. 1921 The principles of immunology. Lippincott, Philadelphia.

KITASATO, S. 1889 Ueber den Rauschbrandbacillus und sein kulturverfahren. Zeitschr. f. Hyg., **6**, 105–116.

KITASATO, S. 1890 Ueber das Wachstum des Rauschbrandbacillus in fester Nährsubstraten. Zeitschr. f. Hyg., **8**, 55–61.

KITASATO, S., AND WEYL, TH. 1890 Zur Kenntnis der Anaeroben. Zeitschr. f. Hyg., **8**, 41–48.

KELSER, R. A. 1918 Improved methods of immunization against symptomatic anthrax. Jour. Agric. Research, **14**, no. 6, 253–262.

KELSER, R. A. 1923 The identification of Bacillus botulinus and its toxin in culture and in canned food by serological methods. Am. Jour. Pub. Health, 13, 366–376.

KOEGEL, A. 1920 Untersuchungen über das vorkommen von normalpräzipitin gegen Rauschbrand in Blutserum verschiedener Tierarten. Zeitschr. f. Inftionskr. d. Haust., **20**, 351–357; Abst. Bact., **5**, 223.

KOJIMA, KATZUMI 1923 Beiträge zur Erforschung der Rauschbranderreger. 1 Mitteilung. Die Toxinbildung des Typus Foth und die toxicologischen, immunisatorischen und biochemischen Eigenschaften des Toxins. Zeitschr. f. Immunit. und Expt. Therap., **37**, 170–184.

KOJIMA, KATZUMI 1923 Beiträge zur Erforschung der Rauschbranderreger. 2 Mitteilung. Ueber Toxin und Antitoxin der Typen Foth und Kitt und ihre besonderen Verschiedenheiten. Zeitschr. f. Immunit. und Expt. Therap., **37**, 185–202.

KÖVES, J. 1914 Zur Aetiologie des sogenanten Rauschbrandes der Schweine. Berl. tier. Woch. no. 8, p. 134. Cent. f. Bact., Abt. 1, Ref., **62**, 88.

KÖVES, J. Rauschbrand und Bradsotähnliche Krankheit der Schweine. Cent. f. Bact., Abt. 1, Orig., **80**, 40–65.

LANDAU, HANS 1917 Untersuchungen über Gasbrand-und Rauschbrandbazillen, mit besondere Berücksichtigung ihres serologischen Verhältnis und ihren Veränderlichkeit. Cent. f. Bakt., Abt. 1, Orig., **79**, 417–425.

LECLAINCHEE, E., AND VALÉE, H. 1900 Recherches experimentales sur le charbon symptomatique. Ann. de l'Inst. Pasteur, **12**, 202–223, 513–534.

LECLAINCHEE, E., AND VALÉE, H. 1902 Recherches expérimentales sur le charbon symptomatique. Ann. de l'Inst. Past., **14**, 931–939; Bulletin Past., **1**, 44.

LECLAINCHEE, E., AND VALÉE, H. 1913 Sur la vaccination contre le charbon symptomatique. Comptes Rend. Acad. des Sciences, **156**, 989–992.

LECLAINCHEE, E., AND VALÉE, H. 1923 Sur la vaccination contre le charbon symptomatique par les toxines. Comptes Rend. Acad. des Sciences, **176**, 207–210.

LESAGE, J., AND POMMIER, G. 1913 Charbon symptomatique des brebis aprés l'anelage. Bull. soc. cent. med. vet. May 30, 1913, p. 215; Bull. Past., **11**, 644.

LIBORIYS, PAUL 1886 Beiträge zur Kenntnis des Sauerstoffbedürfnisses der Bacterien. Zeitschr. f. Hyg., **1**, 115–176.

MACCRUDDEN, FR. 1910 The products resulting from the putrefaction of fibrin by clostridium salus and Rauschbrand. Jour. Biol. Chem., **8**, 109–114.

MCINTOSH, JAMES 1917 The classification and study of the anaerobic bacteria of war wounds. Nat. Health Ins. Med. Research Comm. Special Report No. 12, H. M. Stationary Office, London, pp. 74.

MARKOFF, WLADIMIR N. 1911 Vergleichende bakteriologische und serologische Studien über Rauschbrand und Pseuderauschbrand. Cent. f. Bakt., Abt. 1, Orig., **60**, 188–222.

Medical Research Committee. National Health Insurance. 1919 Special Report 39. Reports of the committee upon anaerobic bacteria and infection. London, pp. 182.

MARSH, H. 1919 An outbreak of blackleg in sheep. Jour. Amer. Vet. Med. Assoc., **14**, 43–47.

MARSH, H. 1923 Blackleg aggressin in sheep. Jour. Amer. Vet. Med. Assoc., **16**, 100–101.

MEYER, K. F. 1915 The etiology of "Symptomatic Anthrax" in swine. Jour. Inf. Dis., **17**, 458–496.

MIESZNER, H. 1913 Die Anwendung des Dialysierverfahrens nach Abderhalden zur diagnose der Trächtigkeit und von Infektionskrankheiten. Deutsch. tierärztl. Woch., p. 417; Cent. f. Bakt., Abt. 1, Ref., **59**, 208.

MÖLLER, O. 1911 Glykogen und seine Spaltprodukte in Beziehung zum Rauschbrand. Berl. tieraratl. Woch. No. 7, p. 117; Cent. f. Bakt., Abt. 1, Ref., **50**, 454.

NICOLLE, M., CESARI E., AND RAPHAEL, A. 1915 Études sur le Vibrion Septique et let "Bacterium Chauvoei." Ann. de l'Inst. Pasteur, **29**, 165.

NIELSON, IVAR. 1890 Ein Stück moderner Bakteriologie aus dem 12 Jahrhundret. Cent. f. Bakt., Abt. 1, **7**, 267–271.

NITTA NEOSHI 1918 Investigations on blackleg immunization. Jour. Amer. Vet. Med. Assoc., **6**, 446–481.

NORGAARD, VICTOR A. 1898 Blackleg in the United States and the distribution of vaccine by the Bureau of Animal Industry. In 15th Ann. Rpt. Bur. An. Ind., pp. 27–81.

PASTEUR AND JOUBERT 1877 Charbon et Septicémie. Comptes Rend. Acad. des Sci., **85**, 102–115.

PFEILER, W. 1918 Die Erkennung der bakteriellen Infektionsktankheiten mittels der Präzipitationsmethode. Berlin. Cent. f. Bakt., Abt. 1, Ref., **68**, 141.

PFUHL, E. 1907 Die Züchtung anaerober Bakterien in Leberbouillon, sowie in Zuckerbouillon und in gewöhnlicher Bouillon mit einem Zusatz von Platinschwamm oder Hepin unter Leftzutritt. Cent. f. Bakt., Abt. 1, Orig., **44**, 378–383.

RATZ, ST. VON 1913 Die empfanglichkeit der Schwiene füt Rauschbrand. Zeischr. f. Infektionskr., **14**, 8; Bull. Past., **12**, 67.

RAVENNA, E. 1920 Endocardite destra e arterite della polmonare nel carbonchio sintomatico. La Clinica Vet., **43**, 1–46; Veterinary Rev., **4**, 276.

REDDISH, GEORGE F., AND RETTGER, LEO F. 1924 A morphological, cultural and biochemical study of representative spore-forming anaerobic bacteria. Jour. Bact., **9**, 59–78.

REUTER 1916 Gasbrand, Geburtsrauschbrand und Pferderauschbrand. Deutsche tierärztl. Woch., p. 348; Cent. f. Bakt., Abt. 1, Ref., **67**, 72.

REUTER 1916 Gasbrand und Geburtsrauschbrand. Ebenda, p. 335; Cent. f. Bakt., Abt. 1, Ref., **67**, 72.

RICHTER, JOSEF 1916 Die Wissenschaftlichen Grundlagen der simultanen Impfmethode gegen den Rauschbrand der Rinder. Oster. Woch. f. Tierheil., 1913, p. 3; Cent. f. Bakt., Abt. 1, Ref., **66**, 127.

RONCA, V. 1920 Il carbonchio sintomatico nelle vie digerenti. La Clin. Vet., **43**, 195–210; Vet. Rev., **4**, 365.

RONCA, V. 1920 Richerche ematologische nel carbonchio sintomatico sperimentale. La Clin. Vet., **44**, 676–694; Abst. Bact., **6**, 383.

RONCA, V. 1921 Sulle alterazione provacate dalle tossine del carbonchio sintomatico. Clin. Vet., **44**, 617–634; Abst. Bact., **6**, p. 367.

ROUX, E. 1887 Sur la culture des microbes anaerobies. Ann. de l'Inst. Past., p. 49; Cent. f. Bakt., Abt. 1, **2**, 327.

ROUX, E., AND CHAMBERLAND, CH. 1887 Immunite contre la septicémie conferée per des substances solubles. Ann. de l' Inst. Past., no. 12, 561; Cent. f. Bakt., Abt. 1, **3**, 769.

ROUX, E. 1888 Immunite contre le charbon symptomatique conferée par des substances solubles. Ann. de l' Inst. Past., no. 2, 49.

SCHATTENFORH, A. 1903 Chemische-biologische Verhalten des Rauschbrandbacillus und des Oedembacillus. Arch. f. Hyg., **48**; Bull. Past., **1**, 720.

SCHMITT 1918 Rauschbrand beim Fohlen. Berl. tierarztl. Woch., 1918, 25; Cent. f. Bakt., Abt. 1, Ref., **69**, 500.

SCHÖBL, OTTO 1910 Ueber Aggressinimmunisierung gegen Rauschbrand. Cent. f. Bakt., Abt. 1, Orig., **56**, 395–399.

SCHÖBL, OTTO 1912 Weitere Versuche über Aggressinimmunisierung gegen Rauschbrand. Cent. f. Bakt., Abt. 1, Orig., **62**; 296–304.

SCOTT, J. P. 1922 The production and potency of antiblackleg serum. Vet. Al. Quarterly, Ohio State Univ., **10**, 47–53.

SCOTT, J. P. 1923 Notes on blackleg immunization. North Amer. Vet., **4**, 267–270.

SCOTT, J. P. 1923 Blackleg vaccines, their production and use. Kan. Agric. Expt. Sta. Tech. Bull. No. 10, pp. 24.

SCOTT, J. P. 1923 Potency tests for blackleg filtrate and aggressin based on the aggressive action of these products. Jour. Amer. Vet. Med. Assoc., **17**, 67–74.

SPIEGEL, A. 1920 Untersuchungen über den Rauschbrand der Schafe. Deutsche tierärztl. Woch., no. 28, 467–470; Abst. Bact., **5**, 225.

STEINBRÜCKE 1918 Kriegesfahrungen über die Beziehungen des Gasbrandes des Menschen zum Rauschbrand der Tiere. Berl. Tierärztl. Woch. 1918, p. 431; Cent. f. Bakt., Abt. 1, Ref., **70**, 196.

SZASZ, A. 1918 Kann man die Rinder gegen Milzbrand und Rauschbrand gleichzeitig (simultan) impfen? Zeitschr. f. Infektionskr. d. Haust., 375; Cent. f. Bakt. Abt. 1, Ref., **69**, 468.

VEILLON, A, AND MAZÉ, P. 1910 De l'emploi des nitrates pour la culture et l' isolament des microbes anaerobies. Comptes Rend. Soc. Biol., **48**, 112; Bull. Past., **8**, 344.

WARNECKE, H. 1917 Muscle lesions in claves resembling blackleg, caused by a coli-like bacillus. Tijdachr. v. Diergenseesk, **44**, 537–530; Abst. Bact., **5**, 41.

WASSERMAN, H. VON 1916 Experimentell-therapeutische Studien aus der Grüppe der Gasbranderreger. Med. Klinik, p. 437; Cent. f. Bakt., Abt. 1, Ref., **66**, 277.

WEINBERG, M. AND SÉGUIN, P. 1918 La Gangrene Gazeuse. Monographie de L'Institut Pasteur, Paris.

WERNE, G. 1905 Die agglutinationation bei Gasphlegmonbazillen. Arch. f. Hyg., **53**, 128; Bull. Past., **3**, 651.

WULFF, F. 1912 Über Rauschbrand und rauschbrandähnliche Erkrankungen. Deutsche tierärztl. Woch., p. 609, 625; Cent. f. Bakt., Abt. 1, Ref., **55**, 501.

WULFF, F. 1912 Die Rauschbranddiagnose durch untersuchung der Galle. Deutsche tierarztl. Woch., p. 705; Cent. Bakt., Abt. 1, Ref. **56**, 367.

ZEISZLER, JOHANNES 1919 Der Rauschbrand und verwandte Erkrankungen der Tiere. Berl. Klin. Woch., 488; Cent. f. Bakt., Abt. 1, Ref., **70**, 197

ZEISZLER, JOHANNES 1920 Menschliche Wundinfektionen und Tierseuchen. Zeitschr. f. Infekt. Parasit. Krank. u. Hyg. d. Haust., **21**, 1–56.

Pag
Mi's

ge

ssing

RECENT WORK ON THE DESCRIPTIVE CHART AND THE MANUAL OF METHODS

H. J. CONN, *Chairman*, K. N. ATKINS, J. H. BROWN. B. COHEN, G. J. HUCKER, F. W. TANNER, *Committee on Bacteriological Technic*

Received for publication March 16, 1925

A descriptive chart for the pure culture study of bacteria, as is well known, has been published by the Society of American Bacteriologists for about twenty years. During this time it has been revised frequently, the most recent revision until that of the past year being the one undertaken in 1920. For the last two or three years a loose leaf hand-book known as the Manual of Methods for Pure Culture Study has also been published by the Society to accompany the chart. The present report is written to give the Society members information as to recent developments regarding both of these publications.

As it is planned to revise the descriptive chart at least every four or five years, it was felt that a revision during 1924 would be desirable. Accordingly opinions as to the weak points of the 1920 chart were secured from as many users of the chart as were interested enough to contribute their opinions; and a chart revised along the lines that seemed to be indicated was submitted to the committee members in the fall of 1924. After obtaining the comments and criticisms from the different members of the committee a new chart was drawn up and submitted to the Society at its Washington meeting December 29, 1924. It is now ready for printing and will have been put on sale by the Society before this article appears in print.

A copy of the new chart accompanies this article and can be compared with the 1920 chart by any one having a copy of the same available. In general plan the chart is unchanged but it has been considerably improved in detail. One of the important changes is to remove the name Descriptive Chart from the top

so that the data concerning the history and isolation of the organism may be placed at the very top of the chart. The terminology has been slightly improved throughout the chart; and more space has been allowed for recording the size of vegetative cells and spores as well as staining reactions. A section has been introduced for recording the optimum H-ion concentration and the pH limits for growth; more columns have been inserted for the fermentation of unspecified carbohydrates; and at the same time certain optional spaces have been introduced into the margin of the chart. Another change in the margin of the chart has been made in that it is proposed to use letters instead of figures to indicate characteristics that are variable, doubtful or undetermined. Except for these changes and a few others of like nature the chart is unaltered. To anyone unfamiliar with its use the new chart appears almost like the old.

While this revision of the chart was being kept in mind, work was also being done to keep the material in the Manual of Methods up-to-date. This Manual of Methods was first issued in February, 1923. It was drawn up as carefully as possible; but the very nature of the material in it made it difficult to include the best and latest information in all cases. Such material as that in this publication may easily be out of date before it is actually published. As a result, before the year of issue was over certain sections already in need of revision were called to the attention of the committee. As the committee had obtained permission from the Society, when the Manual was first proposed, to revise sections of it from time to time, provided the revisions were not such as to change radically the methods given in it, certain sections were rewritten during 1924 and distributed to those holders of the Manual who had entered subscriptions to have their copies kept up-to-date. The sections that have been revised up-to-date are as follows:

The section dealing with preparation of media was revised in January, 1924, principally so as to include practical instructions for preparing media containing the new indicators brom-cresol green and brom-chlor-phenol blue. At the same time the material previously published in this section was rearranged so as to make its use more convenient.

The section dealing with morphology was revised in February, 1924, largely to include the results of recent committee investigations on the Gram stain.

The section dealing with fermentation of sugars and other similar substances was revised in January, 1924, not only so as to rearrange the material in more practical form, but also to give the results of recent information showing practical methods of using indicator media to determine acid production.

The sections dealing with nitrate reduction, hydrolysis of starch and indole-production were revised in October, 1924 so as to include references to certain methods either recently described or recently called to the attention of the committee. These methods are now published in the folders dealing with the subjects in question; but it is not proposed to substitute them for the old methods. The old methods are still given with the suggestion that both the old and the new procedures be compared. For this reason it did not seem necessary to submit the new methods to the Society for approval before referring to them in the Manual.

A very complete revision was undertaken of the section dealing with the determination of hydrogen-ion concentration, and the new folder issued in September, 1924. In this case also no new methods were given in place of the old methods; but the discussion was completely rewritten. When the first version was written, the subject of H-ion concentration was less familiar to bacteriologists than it is at the present day. For this reason it has proved possible to leave out some of the introductory material and to include in its place certain data of value in hydrogen-ion determinations. Particularly useful are Clark's tables of buffer mixtures, which are now given in full in this section of the Manual.

The chart and Manual are now quite widely used in both American and foreign institutions. The sales of the Descriptive Chart now total about 30,000 a year while about 1000 copies of the Manual of Methods have been sold during the two years since it was first published. These sales have been to institutions and private individuals located all over the United States and also

in Canada, England, Holland, Denmark, Australia, China, and Japan. This widespread use suggests that these publications must be filling a real need, and that the Society is accomplishing a worth-while purpose in issuing them.

In just one way it is possible that instructors are not taking complete advantage of these publications. When the Manual was first issued it was felt that in cases where the chart is used by classes of considerable size the instructor might wish to have a copy of the Manual of Methods in the hands of each student using it. In such cases as these the manual would be used only one season and a permanent copy would not be desired. To meet such a demand as this the committee arranged to publish it in paper cover as well as in the regular imitation leather cover. This paper-covered form of the manual is sold for only 50 cents apiece or only 40 cents each when 10 or more copies are ordered. During 1923 quite a number of institutions ordered the paper-covered manuals to be used in this way; but there have been fewer such orders since then. It is felt that possibly instructors do not realize in general that it can be obtained in this cheaper form when desired for student use. For that reason the present occasion is taken to call attention again to this fact.

In conclusion attention must be called to the fact that this chart and Manual are coöperative undertakings in which it is hoped that the Society as a whole may have some part. Care has always been taken to see that these publications do not commit the Society to opinions which are actually personal opinions of members of the committee and that methods which happen to appeal to committee members are not listed to the exclusion of all others. For this reason any method that proves workable in the pure culture study of bacteria is welcomed by the committee and will be gladly inserted in future editions of the Manual of Methods. The loose-leaf form of publication makes it very easy to do this. The committee is glad not only to obtain suggestions of this sort but also to receive criticisms. No one on the committee has infallible judgment nor has had experience with all the types of bacteria for which these methods should apply; and although advice has been asked and received from various

quarters before drawing them up into their present form, it is recognized that they are still far from perfect For this reason no criticism is resented by the committee and any user of the chart finding the present form unworkable or the present Manual of Methods unsatisfactory for his purpose is invited to communicate with the chairman (H. J. Conn, Agricultural Experiment Station, Geneva, New York). It stands to reason that no tests of the chart or of the methods in the Manual which the committee members can give in their own laboratories will be as rigorous as that of actual use in instruction laboratories throughout the country. Hence the committee hopes for the continued coöperation of all users in making both of these publications better in the future.

Name of organism..... Habitat .....

Date of isolation..... History .....

MORPHOLOGY

Underscore required terms. | SKETCHES

VEGETATIVE CELLS, Medium used.....
reaction.....temp.....age.....days.
Form, *spheres, short rods, long rods, filaments, commas, short spirals, long spirals, curved.*
Arrangement, *single, pairs, chains, fours, clusters, cubical packets.*
Limits of length.....; of diameter.....
Size of Majority.....
Ends, *rounded, truncate, concave, tapering*.....
CAPSULES, present on.....
How stained.....

SPORANGIA, *present, absent.* Medium used.....
reaction.....temp.....age.....days.
Form, *elliptical, short rods, spindled, clavate drumsticks.*
Limits of length.....; of diameter.....
Size of Majority.....
ENDOSPORES, *present, absent.*
Method of examination, in *stained* or *unstained* preparations.
If stained, by what technic?.....
.....
Location of Endospores, *central, excentric, subterminal, terminal.*
Form, *spherical, elliptoid, cylindrical*
Limits of Size.....
Size of Majority.....
Wall, *thick, thin.*
Sporangium wall, *adherent, not adherent.*

MOTILITY
In broth..... On agar.....
FLAGELLA, No. .....Attachment, *polar, bipolar, peritrichiate.* How stained.....

IRREGULAR FORMS.
Present on.....in.....days at .....°C.
Form *spindled, caneate, filamentous, branched.* or.....

STAINING REACTIONS.
Gram: 1 day.....; 2 day.....; 3 day.....; 4 day.....
Technic used.....day.....
.....
Acid fast.....
Special stains.....
.....
.....
.....

CULTURAL CHARACTERISTICS

Underscore required terms | SKETCHES | Underscore require

Agar Stroke

Growth, *scanty, moderate, abundant, none.*
Form of growth, *filiform, echinulate, beaded, spreading, arborescent, rhizoid.*
Elevation of growth, *effuse, flat, raised, convex.*
Lustre, *glistening, dull.*
Surface, *smooth, contoured, rugose.*
Optical Characters, *opaque, translucent, opalescent, iridescent.*
Chromogenesis.....Photogenic. Fluorescent.
Odor, *absent, decided, resembling*.....
Consistency, *butyrous, viscid, membranous, brittle.*
Medium, *grayed, browned, reddened, blued, greened, unchanged.*

Incubation Temperature

.....°C

Age

.....d

Gelatin Stab

Growth, *uniform, best at top, best at bottom.*
Line of puncture, *filiform, beaded, papillate, villous, arborescent.*
Liquefaction, *none, crateriform, infundibuliform, napiform, saccate, stratiform;* begins in.....d. complete in.....d.
Degree of liquefaction in.....days.....
Method used.....
.....
Medium, *fluorescent, browned, unchanged.*

Temperature

.....°C

Age

.....d

Medium (solid)

Temperature

.....°C

Age

.....d

Nutrient Broth

Temperature

.....°C

Age

.....d

Surface growth, *membranous,*
Clouding, *slig transient, pers*
Odor, *absent, de*
Sediment, *comp flaky, viscid o*
Amount of sedi *none.*

Medium (liquid)

Temperature

.....°C

Age

.....d

Medium

Temperature

.....°C

Age

.....d

*Recording the "Index Number" here is optional; but its use will be found convenient if the charts are to be filed ac
(primary characteristics) copied down in the order of their occurrence in the margin, placing a dash wherever a heavy ru

Studied by ..... Culture No. .....

Classification according to optimum conditions: ..... Series No. .....

Index No.* .....

BRIEF CHARACTERIZATION

* As each of the following characteristics is determined, indicate in proper marginal square by means of figure, as designated below. In case any of these characteristics are doubtful or have not been determined, indicate with the letter U, V, and X according to the following code:
U, undetermined.
V, variable.
X, doubtful.

PRIMARY CHARACTERISTICS

Microscopic Features

- Form: 1, streptococci; 2, diplococci; 3, micrococci; 4, sarcinæ; 5, rods; 6, commas; 7, spirals; 8, branched rods; 9, filamentous
- Endospores: 0, absent; 1, central; 2, excentric to terminal
- Flagella: 0, absent; 1, peritrichic; 2, polar
- Gram stain: 0, negative; 1, positive

Miscellaneous Biochemical Reactions

- Biologic relationships: 1, pathogenic for man; 2, for animals but not for man; 3, for plants; 4, parasitic but not pathogenic; 5, saprophytic; 6, autotrophic
- Relation to oxygen: 1, strict aerobe; 2, facultative anaerobe; 3, strict anaerobe
- Gelatin liquefaction: 0, negative; 1, positive.
- In nitrate media: 0, neither nitrite nor gas; 1, both nitrite and gas; 2, nitrite but no gas
- Chromogenesis: 1, fluorescent; 2, violet; 3, blue; 4, green; 5, yellow; 6, orange; 7, red; 8, brown; 9, pink; 0, none

Carbohydrate Reactions

- Diastatic action: 0, negative; 1, positive
- From d-glucose: 0, no acid; 1, acid and gas; 2, acid without gas
- From lactose: 0, no acid; 1, acid and gas; 2, acid without gas
- From sucrose: 0, no acid; 1, acid and gas; 2, acid without gas

SECONDARY CHARACTERISTICS

Vegetative Cells

- Diameter: 1, under 0.5μ; 2, between 0.5μ and 1μ; 3, over 1μ
- Length: 1, less than 2 diameters; 2 more than 2 diameters
- Chains (4 or more cells): 0, absent; 1, present
- Capsules: 0, absent; 1, present

Spores

- Shape: 1, spherical; 2, ellipsoid to cylindrical
- Diameter: 1, less than diameter of rod; 2, greater than diameter of rod

Cultural Features

Agar Stroke
- Growth: 0, absent; 1, abundant; 2, moderate; 3, scanty
- Lustre: 1, glistening; 2, dull
- Surface: 1, smooth; 2, contoured; 3, rugose

- Agar colonies: 1, punctiform; 2, circular (over 1 mm. diameter); 3, rhizoid; 4, filamentous; 5, curled; 6, irregular
- Gelatin colonies: 1, punctiform; 2, circular (over 1 mm.); 3, irregular; 4, filamentous

Milk
- Acid: 0, no acid; 1, sufficient for curdling; 2, insufficient for curdling
- Rennet curd: 0, absent; 1, present
- Peptonization: 0, absent; 1, present

- Indole production: 0, negative; 1, positive

(Optional spaces)

derscore required terms

urface growth, *ring, pellicle, flocculent membranous, none.*

Clouding, *slight, moderate, strong transient, persistent, none, fluid turbid*

dor, *absent, decided, resembling* .....

ediment, *compact, flocculent, granular, flaky, viscid on agitation.*

Amount of sediment, *abundant, scanty, none.*

re to be filed according to the salient characteristics of the organisms. The Index Number consists of the first thirteen figures from the margin ever a heavy rule occurs in the margin. Thus the colon organism (communis type) has the Index No. 5010—V2020—1110.

Culture No. .....

Agar Colonies		Sketches	Gelatin Colonies		Sketches
Temperature ....°C	Growth, *slow, rapid.*		Temperature ....°C	Growth, *slow, rapid.*	
Age ....d	Form, *punctiform (i. e. under 1 m.m. diam.), circular (i. e. over 1 m.m. diam.), filamentous, irregular, rhizoid.*		Age ....d	Form, *punctiform, circular, irregular, filamentous.*	
	Surface, *smooth, rough, concentrically ringed, radiately ridged.*			Elevation, *flat, raised, convex, pulvinate, crateriform (liquefying).*	
	Elevation, *effuse, flat, raised, convex, pulvinate, umbonate.*			Edge, *entire, undulate, lobate, erose, filamentous, curled.*	
	Edge, *entire, undulate, lobate, erose, filamentous, curled.*			Liquefaction, *cup, saucer, spreading.*	
	Internal structure (low power of microscope), *amorphous, finely-, coarsely-granular, filamentous, curled concentric.*			Internal structure, *amorphous, finely-, coarsely-granular, filamentous, curled, concentric.*	

PHYSIOLOGY

TEMPERATURE RELATIONS

Optimum temperature for growth ....°C.
Maximum temperature for growth ....°C.
Minimum temperature for growth ....°C.

RELATION TO REACTION OF MEDIUM

Medium used .....
Optimum H-ion conc., *about* pH = ...
Limits of pH for growth: *from* ..... *to* .....

CHROMOGENESIS

Nutrient gelatin .....
Nutrient agar .....
Potato .....
.....

PRODUCTION OF INDOLE

Medium: .....
Test used .....
Indole *absent, present in* .....*days*

PRODUCTION OF HYDROGEN SULFIDE

Medium: .....
Test used .....
H_2S *absent, present in* .....*days.*

RELATION TO OXYGEN

Method used .....
Medium ..... Temperature ....°C.
Aerobic growth: *absent, present, better than anaerobic growth.*
Anaerobic growth: *absent, occurs in presence of d-glucose, of sucrose, of lactose, of nitrate; better than aerobic growth.*
Additional data .....
.....
.....
.....

MILK
(with or without indicator)

Temperature ....°C.

Reaction:	1 day	2 days	4 days	7 days	10 days
Acid curd:	1 day	2 days	4 days	7 days	10 days
Rennet curd:	1 day	2 days	4 days	7 days	10 days
Peptonization:	1 day	2 days	4 days	7 days	10 days

Indicator in milk (if any) .....
Influence of indicator on growth .....
Reduction of litmus begins in .....days; ends in .....days; of methylene blue in .....hours

NITRATE REDUCTION

Medium ..... Temperature ....°C.					
Nitrite:	1 day	2 days	4 days	7 days	10 days
Gas:	1 day	2 days	4 days	7 days	10 days
Medium ..... Temperature ....°C.					
Nitrite:	1 day	2 days	4 days	7 days	10 days
Gas:	1 day	2 days	4 days	7 days	10 days

HYDROLYSIS OF STARCH

Breadth of clear zone on starch agar plates.
In .....days: .....
Additional data (e. g., by method of Meyer and Gottheil) .....
.....
.....

FERMENTATION

Temperature ....°C

Medium containing ..... and:	d-GLUCOSE (DEXTROSE)	LACTOSE	SUCROSE				
Gas							
First appearance of acid							
First appearance of alkali							
Reaction after .....days							
Reaction after .....days							
Max. H-ion Conc							

SPECIAL TESTS (e. g. PATHOGENICITY)

This DESCRIPTIVE CHART prepared by the Committee on Bacteriological Technic of the SOCIETY OF AMERICAN BACTERIOLOGISTS. Presented at the annual meeting of the Society, Dec. 29, 1934.
Members of committee: H. J. CONN, K. N. AYERS, J. H. BROWN, BARNET COHEN, G. J. HUCKER, and F. W. TANNER.

STUDIES ON THE METABOLISM OF B. BOTULINUS IN VARIOUS MEDIA. XXVI

E. WAGNER AND K. F. MEYER IN COÖPERATION WITH C. C. DOZIER

From the George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco[1]

Received for publication, October 6, 1924

The observations to be detailed in this paper form a part of a broad program dealing with the biology of *B. botulinus*. The work was begun at a time when little or no data on the metabolism of this anaerobe had been published. Although the chemistry of the waste products formed by *B. botulinus* in different media appeared important, the elucidation of the mechanism of the toxin production was even more fascinating. A number of English workers have carefully investigated the chemical activities of several sporulating anaerobes, but the metabolism of the toxin-producing *B. tetani* and *B. botulinus* has not been studied. Irrespective of the fact that Kendall and associates, De Bord and Schübel have since reported their chemical investigations on *B. botulinus* it was felt that the problem originally formulated should be carried through in order to lay a foundation for some investigations on the nature of the poison. It is needless to emphasize that *B. botulinus* is excellently suited for such an inquiry on account of the great virulence of the toxin produced in practically every medium and on account of the enormous potency of the bacterial ferments elaborated by the organism. The multitude of data thus far collected must be considered as preliminary; the interpretation of a number of experiments may be changed as the work progresses. However, an account of what has been accomplished, incomplete as it may be, appears fully justified.

[1] This study was aided by grants from the National Canners Association, the California Olive Association and the Canners League of California.

STUDIES ON THE METABOLISM OF B. BOTULINUS IN VARIOUS MEDIA. XXVI

E. WAGNER AND K. F. MEYER IN COÖPERATION WITH C. C. DOZIER

From the George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco[1]

Received for publication, October 6, 1924

The observations to be detailed in this paper form a part of a broad program dealing with the biology of *B. botulinus*. The work was begun at a time when little or no data on the metabolism of this anaerobe had been published. Although the chemistry of the waste products formed by *B. botulinus* in different media appeared important, the elucidation of the mechanism of the toxin production was even more fascinating. A number of English workers have carefully investigated the chemical activities of several sporulating anaerobes, but the metabolism of the toxin-producing *B. tetani* and *B. botulinus* has not been studied. Irrespective of the fact that Kendall and associates, De Bord and Schübel have since reported their chemical investigations on *B. botulinus* it was felt that the problem originally formulated should be carried through in order to lay a foundation for some investigations on the nature of the poison. It is needless to emphasize that *B. botulinus* is excellently suited for such an inquiry on account of the great virulence of the toxin produced in practically every medium and on account of the enormous potency of the bacterial ferments elaborated by the organism. The multitude of data thus far collected must be considered as preliminary; the interpretation of a number of experiments may be changed as the work progresses. However, an account of what has been accomplished, incomplete as it may be, appears fully justified.

[1] This study was aided by grants from the National Canners Association, the California Olive Association and the Canners League of California.

Since it is known from the work of Long (1920), Waksman, Braun and Cahn-Bronner that the nitrogen acceptable as a food is rather specific for each species of bacteria, and since it is assumed but by no means proven that the toxin is a nitrogen split product, the nitrogen metabolism of *B. botulinus* was carefully investigated. It was equally important to analyze the factors at work and the mode of action of this anaerobe in breaking down the nitrogen containing molecules and to study the degradation products resulting therefrom. It is now believed that the quantitative changes in the amino acids, the ammonia, the non protein nitrogen and the qualitative determinations of other nitrogenous constituents furnish the best and the most readily obtainable index concerning the nitrogen metabolism of an organism. The suitability of a medium for growth is usually judged by its amino acid content (Walker Hall) on account of the prevailing conception that amino acids are used directly for bacterial development (Bainbridge, Rettger *et al.*). Numerous attempts to grow *B. botulinus* in substrata composed of purified amino acids have been futile. This microörganism apparently lacks the ability to synthesize nitrogen groups from single amino acids and ammonium salts. Whether this property is common to the obligate anaerobic bacteria deserves careful investigation. It is not unlikely that, provided the other nutritional needs (carbohydrates, salts, etc.) are properly satisfied, purified amino acids may serve as an adequate nitrogen source. The rapid degradation of the nitrogen molecules to amino acids in cultures of *B. botulinus* suggests that these split-products may form the food sources of the organism. In all probability the amount of amino acid present in a growing culture at any one time represents the excess over what is used by the bacteria. Furthermore the amino nitogen may be taken as a criterion to determine the degree of proteolysis, but independently it is an unreliable index for the estimation of nitrogen disintegration.

Preliminary tests have shown that ammonia is the chief nitrogenous end product of the metabolism of *B. botulinus*. In this sense, urea excreted in the urine of mammals and ammonia in cultures of bacteria have an almost parallel significance.

Kendall's (1911) conception is "that the accumulation of free ammonia in cultures of bacteria is very largely, if not exclusively, the result of intracellular utilization of protein or protein derivatives for structural requirements and more particularly for energy." There is little evidence to support Doryland's extreme view that ammonia is the starting point for bacterial development. Non-protein nitrogen is considered the most reliable measure of proteolysis; this subject has been discussed in a former paper.

Disagreement exists concerning the ability of *B. botulinus* to ferment carbohydrates other than glucose. A review of this phase of the chemical activity of *B. botulinus* has been made by Hall (1921) and Kahn and Bengtson (1923).

Little is known concerning the gas production of *B. botulinus*. Van Ermengem thought gas and toxin were related. Hall's data indicate that the presence of gas is dependent upon carbohydrate utilization. Wolf and Harris (1918) in their study of *B. histolyticus* and *B. sporogenes*, bacteria in many ways similar to *B. botulinus*, found that the organisms produced considerable gas on meat and sugar-free media, 70 per cent of which was CO_2. Information concerning the amount of gas evolved by *B. botulinus* and the time of its spontaneous collection over various media has therefore been secured.

The production of acid by bacteria has been generally accepted as an indication of carbohydrate utilization. Dakin, Newberg, Knoop and others, working with amino acids, have shown that their disintegration products may also give rise to acids. The amino, hydroxy and ketonic acids are closely related and readily change from one form to another. In cultures in which acids are being formed at the expense of carbohydrates, direct titrations may furnish approximate information concerning the amount of acid developed but, in case nitrogenous material is simultaneously utilized, the acids are largely compensated by the formation of basic products, the excess only being ionizable. Pasteur appreciated this fact and relied more upon litmus than upon volumetric determinations of acidity. Methods for the determination of the H-ion concentration of biological solutions

were introduced by Sörensen (1912) and improved by Clark and Lubs (1916) and others. The electrometric determinations of H-ion concentration, which are probably the most accurate for the determination of true acidity, are less convenient than the colorimetric for routine tests.

Kligler (1916), Clark (1916), Wolf (1921) and others have discussed the ability of media to resist changes in pH through the addition of acid or alkali. Brown (1921) has presented a serviceable method, by which this property can be estimated. He has aptly designated it "Buffer Index."

The quantitative determination and the identification of the various acids was considered important. The methods generally used for the estimation of the volatile acids are fairly reliable, but those recommended for their separation and identification are long and tedious. The presence of butyric acid in *B. botulinus* cultures has been mentioned by investigators but never conclusively demonstrated. The members of the volatile acid series differ only in slight gradations; the separation of the individual acids can be effected by repeated fractionations. The Duclaux method and its modifications have been usually employed. Wolf and Telfer practiced the Dyer steam distillation technique and by ingenious procedures estimated the acid formed by *B. sporogenes* in milk as follows: 25 per cent acetic, 11.7 per cent butyric, 16.2 per cent valeric and 41.4 per cent caproic. This procedure was found unsatisfactory by Bushnell and was severely criticised by Upson, Plum and Schott. Although it is known that the methods are unreliable for complex mixtures of acids, it has nevertheless been proved that they are capable of yielding comparative information and they are the best procedures thus far developed. Attention has also been paid to the fixed acids. Fred, Peterson and Davenport working with xylose fermenting organisms and Foster with *Streptococcus hemolyticus* found that over 60 per cent of the acidity was due to the non-volatile, lactic acid. Blanchetière and Aubel have published valuable information concerning the fixed acids formed by *B. fluorescens* and *B. pyocaneus* in media of definite chemical composition. The observations of Wolf and Telfer indicate that the

production of acids by anaerobes is quite different from that of aerobes; only 40 per cent of the acids produced by *B. welchii* and *B. sporogenes* are non-volatile.

METHODS

Preparation of the cultures. The apparatus employed by Wolf and Harris (1917) for the incubation, the collection of gases and the sampling of the cultures of an anaerobic organism responsible for wound infections was imitated in this investigation.

A water bath (fig. 1) maintained at a constant temperature of 37°C. by a thermo-electric regulator and large enough to hold four 2-liter pyrex flasks incubated the cultures.[2] The flasks were fitted with 2-hole rubber stoppers. Through one hole a glass tube reached to the bottom of the receptacle and was used for sampling; through the other were placed the connections needed for the evacuation of the flasks. Two-inch pieces of rubber tubing plugged with cotton were slipped over the outer ends of the glass tubes. The stoppers with their fittings were wrapped in paper and sterilized separately. The amount of the medium, the gas space and the capacity of each receptacle was calculated by weighing (*a*) the empty containers (*b*) the flasks completely filled with water and (*c*) the flasks after they had been emptied, charged with the medium and sterilized. An experimental series was usually set up as follows: The cotton plugs in the medium flasks were cautiously replaced by their rubber stopper and connections. These were wired in place and a generous amount of Imperial Cement was poured around the necks of the flaks. They were arranged in the water bath as shown in (fig. 1). In the tube system connecting the fermentation vessels and the manometer a special type of sterile "moisture trap" (*M.T.*, fig. 1) with an air filter were placed. These "traps" are a great improvement over ordinary cotton filters which quickly become damp and favor the entrance of contaminating organisms. Anaerobiosis of the flasks was secured by the use of a Cenko-Nelson two-stage electric pump connected

[2] Made by the Electric Sales Service Company of Berkeley, Calif.

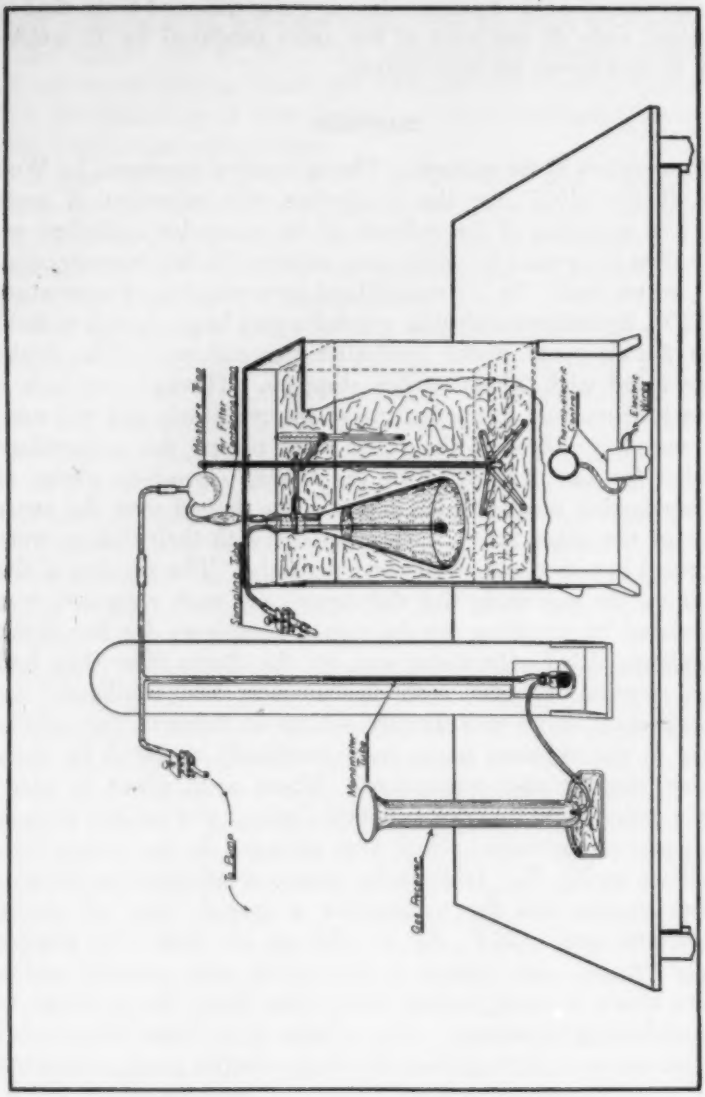


FIG. 1. WATER BATH, GASOMETER, MOISTURE TRAP, ETC., USED FOR THE INCUBATION OF THE CULTURES

through a manometer tube which served as an indicator. It was absolutely necessary to have the best pure gum rubber tubing for all the connections as cheaper grades cracked upon autoclaving and were found unreliable. Shellacking of the tubing and the connections was used to maintain the vacuum. The flasks were inoculated after making sure that the system contained no leaks.

Inoculation. The seed cultures were aspirated into the fermentation flasks. By inserting the large end of a Pasteur pipette into the rubber of the "sampling tube" and the other end into the inoculum the difference in pressure, when the screw clip on the "sampling tube" was cautiously opened, caused the seed culture to be sucked into the medium. The last of the inoculum was carried into the medium by holding the Pasteur pipette in the middle of a Bunsen flame and allowing warm air to be drawn in. The clamp on the rubber of the "sampling tube" was then closed; the pipette removed and the rubber tube washed out with 10 per cent formaldehyde. A piece of cotton was left in the end of the tubing and covered with an ordinary rubber nipple which served as an excellent protector.

Gas reading and calculations. The height of the mercury in the manometer tube, the barometer and temperature readings were taken at the beginning of the experiment and at frequent intervals throughout the gas producing phase of the fermentation. Making use of the equation $V = \frac{273\ V\ (P-T)}{(273\ +\ t)760}$ the amount of gas formed was calculated, due allowance being made, when samples were removed, for the decrease in the medium and the increase in gas space. This form of calculation may be illustrated by an example:

The gas space in the flask, plus that in the connecting tubes is 600 cc. The flask contains 1500 cc. of the culture, the barometer height is 753 mm., the manometer height 400 mm., the temperature 37°C. and the initial vapor tension 40 mm., therefore $600 \times \frac{273\ (753-400-40)}{(273+37)\ 760} \div \frac{1500}{1000} = 513$ cc. of gas per liter.

When large amounts of gas were produced it was collected over water as shown in the illustration. This method is open to criticism on account of the solubility of the gases and other factors; the results, however, are of comparative value.

Sampling and handling of cultures. Previous to the removal of the samples the flasks were agitated to assist in liberating the gases and to mix the cultures thoroughly. Portions of from 50 to 100 cc. were removed through the "sampling tube" with the aid of the pump as described by Wolf and Harris. An extra cotton air filter between the sampling bottle and the pump insured protection to the culture. The amount of material removed was noted and from 5 to 10 cc. were placed in sterile centrifuge tubes for toxin and purity tests; the rest was used for the chemical determinations.

The purity of the cultures was tested either by preparing three dilution shakes cultures in deep liver-agar tubes as described by Heller or by plating the specimens in blood agar. After an incubation period of twenty-four to forty-eight hours the colonies were examined. Contaminated or doubtful cultures were invariably discarded.

Toxin tests were made, either according to the standard method outlined by the Hygienic Laboratory of the United States Public Health Service or by inoculating suitable dilutions of the centrifuged cultures intraperitoneally into either mice or guinea-pigs. In all cases the type of animal used for the tests will be mentioned in the experiments.

ANALYTICAL METHODS

All determinations were made upon live cultures because sterilization by heat or through filter candles produced changes in the nitrogen constituents and the reactions of the cultures. The following methods have been used.

Total nitrogen. A modification of the Kjeldahl procedure was used for total nitrogen determinations. Five cc. of 2 per cent pepton solutions were digested in Kjeldahl flasks with 15 cc. of concentrated H_2SO_4 and a few crystals each of potassium sulfate and copper sulfate for three hours, or until the solutions became

clear and bluish-green in color. The digestion mixture was then cooled, diluted with distilled water, about 1 gram of pumice added to assure even boiling, an excess of NaOH introduced, and the free ammonia distilled into standard acid solutions according to the usual methods.

Non-protein nitrogen. The Folin and Wu precipitant for the removal of proteins from blood was used. Twenty cubic centimeters of 2 per cent pepton (less in case of more concentrated solutions) were accurately measured into a 100 cc. volumetric flask, and diluted to about 70 cc.; 10 cc. of a 10 per cent sodium tungstate solution and—while shaking—$\frac{2}{3}$N H_2SO_4 were added slowly until the reaction was acid to congo-red (approximately 10 cc.); the mixture was then made up to volume. After standing twenty minutes it was filtered through a good grade of filter paper. The filtrate was suitable for estimations of the non-protein nitrogen, creatin, creatinin, uric acid, sugar and other determinations.

For non-protein nitrogen determinations 20 cc. of the filtrate were digested and distilled as for total nitrogen determinations.

Ammonia. Eight-by-1-inch pyrex tubes arranged in series as in the Van Slyke and Cullen apparatus for urea determination were used for ammonia determinations. Five or 10 cc. of culture, depending upon the amount of ammonia suspected, with 3 drops of caprylic alcohol to prevent frothing, were aerated according to the Folin method for two hours with 10 cc. of a saturated solution of Na_2CO_3 (5) into accurately measured amounts of standard acid solution containing alizarin as an indicator. The residual acid was determined by titration and the ammonia was calculated. Duplicate determinations and controls were invariably made with each series.

Amino acids. The Van Slyke method was used for the estimation of the amino acid nitrogen. A three-minute interval was uniformly used for deaminization and corrections for the interference of ammonia were made according to the factors worked out by Van Slyke. The temperature in this laboratory varied slightly from 20°C.; the error involved in using an arbitrary factor for ammonia is therefore not comparable to that found

by Bushnell whose laboratory temperature ranged from 25° to 35°C. In nitrogen-rich media in which the growth of the organism caused a high concentration, the ammonia was removed before the determination of the amino acids.

Creatin and creatinin. The colorimetric methods of Folin and Wu were employed for creatin and creatinin determinations; a pure creatinin standard was used for comparison.

Volatile acids. For the quantitative estimation of volatile acids, the Dyer steam distillation technique was followed. A constant temperature in the distilling flask was maintained by an oil bath in preference to an electric plate. The distilling constants determined for pure acids agreed closely with those established by Dyer so that it can be assumed that the equipment and the manipulations were satisfactory. A detailed account of such tests will be given in the experiments in which identifications of the acids were made. It was found that in the first 500 cc. of the distillate secured from the solutions of known acids, 84 per cent of the acetic, 98 per cent of the propionic and 99.5 per cent of the butyric acids were recovered. Twenty-five to 50 cc. of culture were strongly acidified with 10 per cent H_2SO_4, made up to a volume of 150 cc. and distilled with steam until 500 cc. of the distillate were collected; this was titrated with N/10 standard alkali and reported as cubic centimeters of acid per 100 cc. of culture. In media containing large amounts of volatile acids it was found advantageous to make the determinations on protein-free material; this procedure eliminated the annoyance of frothing and bumping; furthermore it added to the accuracy of the determinations.

Reaction. The H-ion concentration of the cultures was measured electrometrically. All determinations were made by Mrs. Forster whose faithful coöperation and promptness in making the electrometric readings aided much in securing accurate results. Where a high concentration of H_2S prevented the use of the H-electrode, the colorimetric method of Clark and Lubs was used as a substitute. In the tables "E" stands for electrometric and "C" for colorimetric determinations.

Sugar. Folin's (1918) titration method was used for the

glucose determinations. Five grams of a phosphate salt mixture with 5 cc. of a quantitatively prepared copper solution were heated in a large test tube and the sugar-containing solution titrated from a micro-burette, according to the specifications of the method. On account of the dilution of the titration mixture determinations on solutions containing less than 0.1 per cent of sugar were unreliable. The Shaffer method which offers certain advantages was used in some of the later experiments.

Buffer values. The method of Brown (1921) was used to determine the buffer value of a culture medium. The sum of the amount of standard acid to bring the reaction to pH 5.0 (methyl red indicator) and the amount of standard alkali to bring the reaction to pH 8.0 (phenol red indicator) was determined. The figure obtained was termed the "buffer index" and reported as per cent normal.

CHARTING AND RECORDING RESULTS

All results were recorded in terms of 100 cc. of medium, except gas, which was calculated in cubic centimeters per liter. Acidity and alkalinity were reported in terms of N/10 solutions. In the charts the abscissae relate to the media or their split-products, while the ordinates refer to the time.

The data of some of the experiments are recorded in the form of "derived curves." They were prepared as follows: A curve was drawn of the gas production, i.e. the total amount of gas formed in a certain time was plotted against time and the curves for the other products were constructed using the same apex and taking graphic values in such a manner that the final point of each product coincided. Important conclusions may be drawn from curves of this kind; if, for example, the gas and acid are the result of certain chemical changes the production of a known amount of acid will be accompanied by the liberation of a definite amount of gas. In this case, curves showing the amount of acid and gas would coincide.

Hourly production curves were frequently prepared by plotting the amounts of the metabolites formed in the culture during a given period against the time. These curves show clearly the

periods during which the activity of the organisms was most intense.

EXPERIMENTAL DATA

The conception that meat protein is necessary for the elaboration of *B. botulinus* toxin was so prevalent among bacteriologists that as recently as 1921 the fact that *B. botulinus* could grow and produce its toxin in ordinary cottage cheese was reported by Nevin as unusual. This is by no means surprising since previous to this date most of the accounts of the biochemical activity of *B. botulinus* were based on the studies of European investigators who probably worked with organisms similar to, or identical with, Type C *B. botulinus*. This group of bacteria has peculiar nutritional requirements while the common American Types A and B are not very exacting in their protein needs as will be shown in the pages which follow.

Preliminary tests had demonstrated that *B. botulinus* grew and produced its toxin in a 2 per cent pepton solution. Since this medium has the advantage of being sugar-free and is relatively simple in composition, it was decided to begin the study of the metabolic activities of the anaerobe in this solution and later to follow its development in media better suited to bring out characteristic reactions.

The experiments detailed below, unless otherwise stated, were conducted in the following manner. Approximately 2 per cent solutions of Parke-Davis or Difco pepton with 0.5 per cent sodium chloride were prepared with distilled water and adjusted with sodium hydroxide to pH 7.6, distributed in 2 liter flasks and sterilized at 115° for thirty minutes on 2 consecutive days. In the majority of experiments the fermentation of *B. botulinus* in plain pepton solution was compared with that in glucose-pepton. The carbohydrate was invariably added as a sterile solution to the basic medium shortly before inoculation. As a seed culture, 15 cc. of a twenty-four to thirty-six-hour growth of *B. botulinus* in a 1 per cent glucose-veal infusion pepton-broth was aspirated into approximately 1600 cc. of medium.

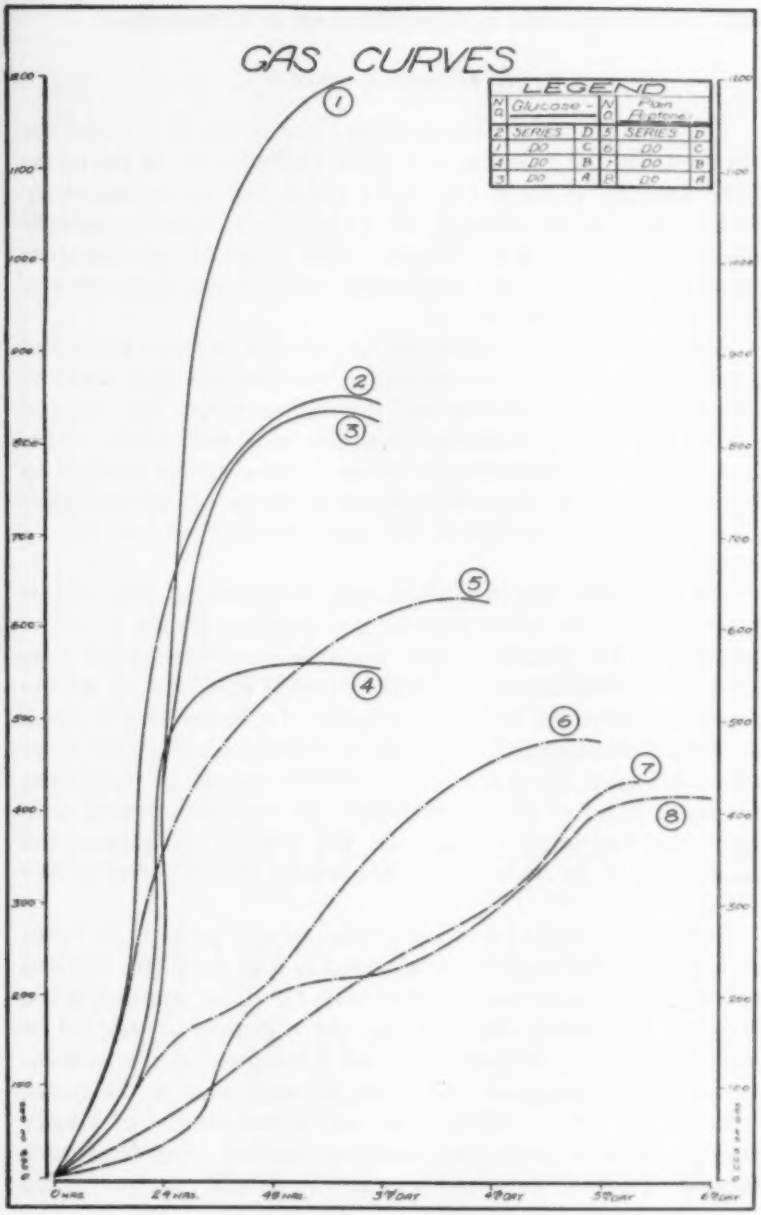


CHART 1. GAS PRODUCTION OF B. BOTULINUS IN PLAIN AND GLUCOSE PEPTON

Experimental series 1

The serologic differences between Type A and B *B. botulinus* toxin evoked the question: Are there dissimilarities in the metabolic activity of these two types which can be measured by biochemical tests? Growth of Type A (a recently isolated strain 38) and Type B (the old Nevin strain 6) was therefore compared in media free from sugar and in one containing a known amount of glucose.

Judging from the turbidity of the samples removed there was good growth in all of the cultures. Gas readings were made at frequent intervals throughout the fermentation and on the fourth day after inoculation chemical tests were made. This interval gave an opportunity for the gross chemical changes to take place and for any differences in inoculums to be equalized. In a subsequent experiment the later chemical changes will be discussed.

The evolution of gas showed early chemical activity and is indicative of the suitability of the medium (chart 1). The amount of gas collected from the glucose cultures after four days incubation was approximately double that evolved by the organism grown in the plain pepton. In Series D the small accumulation of gas can probably be attributed to a slow leak. Gas production in the glucose cultures was at its maximum twenty-four hours after inoculation; the evolution ceased soon after the forty-eighth hour. In the absence of glucose the production of gas was more or less regular over a period of five days.

In this experiment an excess of glucose was present; in Series A, C and D, the bacteria metabolized in four days 510, 670 and 490 mgm. of glucose respectively (table 1). The variation in the amounts of glucose consumed by the organism in this set of experiments may be attributed to differences in the concentration of the peptones (see total nitrogen) and in the buffer value of the media, which according to the studies of Kligler and of others influence the sugar metabolism. Supplementary tests in which *B. botulinus* was grown on a 2 per cent Difco

TABLE 1

Activity of B. botulinus type A and B on pepton with and without glucose

	MEDIA 2 PER CENT SOLUTIONS	TIME	TOTAL N	AMINO N	AMINO N CHANGE	AMMONIA N	AMMONIA FORMED	VOLATILE ACID N/10	pH	SUGAR	GAS PER LITER
			Strain 6, Type B								
			mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*cc.*	"E"	*per cent*	*cc.*
Series A	Parke-Davis pepton	Initial	406	81.0		7.0		1.4	7.71	–	0
		4 day	–	116.8	35.8	106.4	99.4	43.0	7.74	–	422
	Parke-Davis pepton + glucose	Initial	400	95.6		5.3		3.2	7.66	1.25	0
		4 day	–	133.0	57.4	25.2	19.9	29.0	5.31	0.74	812
Series B	Parke-Davis pepton	Initial	380	70.2		7.0		1.2	8.40	–	0
		4 day	–	71.0	0.8	71.0	64.0	30.5	7.40	–	268
	Parke-Davis pepton + glucose	Initial	370	68.0		7.0		1.0	8.40	3.30*	0
		4 day	–	110.0	42.0	46.2	39.2	30.0	6.20	0	550
			Strain 38, Type A								
Series C	Parke-Davis pepton	Initial	305	54.3		3.4		1.4	7.66	–	0
		4 day	–	58.4	3.9	53.8	50.4	29.0	7.60	–	451
	Parke-Davis pepton + glucose	Initial	–	50.7		3.4		2.2	7.64	1.10	0
		4 day		63.6	12.9	18.2	7.8	14.1	5.30	0.43	1180
Series D	Difco pepton	Initial	358	81.7		2.8		4.8	8.00	–	0
		4 day	–	70.4	−11.3	42.8	40.0	34.0	7.08	–	630
	Difco pepton + glucose	Initial	341	79.0		2.8		2.0	7.80	1.14	0
		4 day	–	76.0	−3.0	17.0	14.2	13.8	4.75	0.65	823

* Most of sugar removed in control sample.

pepton with various concentrations of glucose (1 to 6 per cent) demonstrated that irrespective of the sugar present only 550 mgm. of glucose were used in the course of a fermentation period of four days.

Comparison of the total sugar destruction with the total amount of gas which was recovered demonstrated a definite relationship between the two. In Series A and D in which a similar amount of sugar was consumed similar gas accumulations were recorded, in Series C a greater utilization of glucose was accompanied by a corresponding increase in gas production, while in Series B with a small amount of the carbohydrate little gas was formed.

The initial reaction of pH 7.6 in the plain pepton cultures changed little after four days growth. In the presence of sugar as in Series A and C, a pH of 5.3 developed; in Series B, insufficient glucose was apparently responsible for a pH of 6.2. In Series D, a pH of 4.75 developed. Difco replaced the Parke-Davis pepton used in the other series which probably accounts for this variation.

The cessation of the gas production, the definite clearing of the culture fluids and the microscopic appearance of the organisms all indicated that after four days incubation the activity of the glucose cultures was over. It cannot be stated with certainty that the acidity developed was growth-inhibitory since Mrs. Forster, working in this laboratory, has been able to initiate growth of *B. botulinus* in pepton solution at pH 4.4. To elucidate this point, sterile filtrates from five day Difco and Parke-Davis glucose-pepton cultures of *B. botulinus* were reseeded and no growth resulted; the addition of glucose was of no avail; when, however, the filtrate was adjusted with sterile sodium hydroxide to an optimum pH it supported fair growth. It therefore must be concluded that the H-ion concentration was at least one growth limiting factor. Jones (1920) attributed growth cessation of *B. proteus* cultures "to the paralyzing effect of the hydrogen ion concentration on the endoenzymes of the bacteria." While little is known concerning the enzymes of *B. botulinus* the work of Dernby and Blanc and that of Rona and Arnheim

indicate that a reaction of pH 5.3 would be insufficient to inactivate either proteolytic or glycolytic enzymes.

Approximately 20 per cent of the nitrogen in the media was present as amino acid. After four days growth the concentration increased in Series A, B and C. Assuming that nitrogen compounds of the medium are converted into amino acid before utilization an increase indicates that the complex nitrogen molecules were broken down more rapidly than the needs of the bacteria demanded. The presence of glucose favored the accumulation of amino acid; the significance of this fact was discussed in a previous paper (Dozier, Wagner and Meyer) and was mentioned by De Bord.

The ammonia nitrogen in the glucose-free cultures increased in Series A, B, C and D respectively to 99.4, 64, 50.4 and 40 mgm. per 100 cc. of culture. Comparatively small accumulations of ammonia occurred in the glucose cultures and probably represent a minimum use of nitrogenous material. The organisms growing in the glucose-free culture were forced to select for energy needs carbonaceous material from the amino acids, causing a large nitrogen waste which appeared as ammonia.

More volatile acid was present in the plain pepton cultures than in those containing glucose. In Series B, the amount of volatile acid was the same in both cultures; judging from the amino acid and ammonia formation the nitrogen breakdown was also similar. In this series little sugar was present to modify the character of the fermentation. The experimental data are in harmony with the conception that the disintegration of the amino acid compounds give rise to acid products.

The inevitable variability that is encountered in this type of experimental work makes it inadvisable to draw general conclusions; however the amino acid, ammonia and volatile acid changes indicate that strain 6, Type B, was chemically more active than strain 38, Type A. The fact that strain 6 has been isolated for a number of years and has probably become accustomed to artificial media, may in part explain the more vigorous activity reported in this experiment. The results are in agreement with those of De Bord both as to the variability

of growth and as to the increased activity of Type B over that of Type A in 2 per cent pepton solutions.

Experimental series 2

The growth activities of *B. botulinus* in pepton solution were followed by a series of tests in which samples of the culture were frequently removed and analyzed for changes in the nitrogen constituents, in the reactions and in the sugar content. Such data enable the worker not only to detect the maximum changes, but they also offer an opportunity to relate the chemical activities which take place in the cultures. Such correlations are aided by the construction of derived curves.

TABLE 2

Growth of B. botulinus on plain and glucose Parke-Davis pepton

Type A, strain 38

pH	VOLATILE ACID N/10	AMMONIA N	AMINO N	GAS PER LITER	TIME	GAS PER LITER	AMINO N	AMMONIA N	VOLATILE ACID N/10	pH	SUGAR
"E"	*cc.*	*mgm.*	*mgm.*	*cc.*		*cc.*	*mgm.*	*mgm.*	*cc.*	"E"	*per cent*
7.66	1.4	3.4	54.3	0	0	0	58.0	3.4	2.2	7.64	1.10
7.60	3.0	4.9	65.0	146	24 hours	411	56.0	11.0	12.8	6.14	0.69
7.58	—	7.0	66.0	212	48 hours	1135	88.1	20.4	13.4	5.33	0.44
7.6 C	29.0	53.8	58.4	589	96 hours	1180	88.1	—	14.1	5.4 C	0.43
7.79	32.0	61.6	87.0	652	7 days	—	92.0	20.4	14.4	5.34	0.40

Two experimental series were conducted. In one series a Type A strain 38 was grown in a 2 per cent Parke-Davis and in another a Type B strain 53 was cultivated in a 2 per cent Difco pepton. For the sake of brevity only the results of the analysis of the Type A culture are detailed.

After twenty-four hours of incubation 411 cc. of gas was collected from the glucose culture while less than one-third of that amount was formed by the organisms grown in the plain pepton medium (table 2). The change in the reaction and the loss in glucose were further evidences of vigorous activity. In the glucose culture, the maximum chemical changes had occurred

by the forty-eighth hour so that little variation was noted after five days incubation. Gas accumulated continuously over a period of seven days in the sugar-free culture. Between the forty-eighth and ninety-sixth hours 377 cc. of gas and 45.8 mgm. of ammonia were formed. The appearance of spores and the insignificant accumulation of metabolites indicated that the period of active proliferation of the organisms was over. The sluggish increases in ammonia and volatile acids which depict deep seated chemical changes, were probably due to a small number of growing bacteria, while the accumulation of amino acids in old cultures was probably the work of enzymes.

The unfavorable reaction which developed in the course of the fermentation of 700 mgm. of glucose prevented further visible development in the glucose culture. Why growth should apparently be inhibited in the plain pepton culture is not understood. Wolf and Harris (1917) report a similar though more rapid rise and fall of chemical activity of *B. sporogenes* in pepton cultures. The increases in amino acid and ammonia caused by *B. botulinus* are indicative of proteolysis; there undoubtedly was no lack of soluble nitrogenous material in the medium. Since *B. botulinus* is unable to grow in synthetic media containing mono and dibasic amino acids and in this respect differs, for example, from the tubercle bacillus, which is able to synthesize specific nitrogenous compounds, it is reasonable to assume that the exhaustion of certain vitally important amino acids will limit its growth. Furthermore it is recalled that Long (1923, p. 52) considers the compounds, which remain after the deamination of the cyclic amino acids, as growth inhibitory. A similar process may have been at work in the pepton cultures of *B. botulinus*.

The vegetative organisms in both of the experiments made a prompt attack upon the glucose of the medium. When spores were used for inoculation, as in the experiments already reported (Dozier, Wagner and Meyer) the disappearance of the sugar was delayed during the period of germination of the spores. The sparing action, as conceived by Kendall and expressed in a reduced ammonia production was more pronounced in the second

than in the first series. In this connection the studies of Bushnell, on the effect of carbohydrates in 2 per cent pepton solutions on the metabolism of an organism (probably *B. sporogenes*) are of interest. According to his data it is evident that 0.5 per cent glucose failed to exert a sparing action after the first days of incubation, while 1 per cent glycerol had the same influence as a similar concentration of glucose had on the *B. botulinus* cultures under consideration.

There is no agreement among workers concerning the fermentative ability of *B. botulinus*; a discussion of this phase is reserved for a later communication. However, it is well at this time to call attention to experiments in which lactose, sucrose, and soluble starch were added in 2 per cent concentrations to pepton cultures of *B. botulinus*. After an incubation period of four days the figures for the ammonia, the H-ion concentration and the gas volume were as follows:

Strain 38

	AMMONIA NITROGEN	pH	GAS PER LITER
	mgm.		*cc.*
Lactose	82.0	6.8	—
Sucrose	92.4	6.8	835
Starch	53.0	6.2	966

It is evident that these carbohydrates are not readily available for the energy needs of *B. botulinus* and that, with the exception of starch, they apparently exert no sparing action.

Volatile acids were produced in considerably larger amounts in the plain pepton media than in those containing glucose. In the Difco pepton more volatile acids were formed than in Parke-Davis pepton solution. These differences are probably due to the type of acid formed in the cultures; for example, acetic acid is more toxic to bacterial development than the other members of the volatile acid series. It is not unlikely that *B. botulinus* produced primarily acetic acid in Parke-Davis pepton solutions.

The reactions in plain pepton media remained quite constant; in the presence of glucose the reactions quickly became acid and remained so. A higher H-ion concentration (pH 4.7) de-

veloped in Difco than in the Parke-Davis pepton cultures (pH 5.34).

From the derived curves (chart 2) the following inferences may be drawn: In glucose-pepton-cultures, the formation of gas, the sugar consumption and the changes in the reaction are dependent upon each other, the ammonia production, though slight, is inhibited at the same time as the other activities;

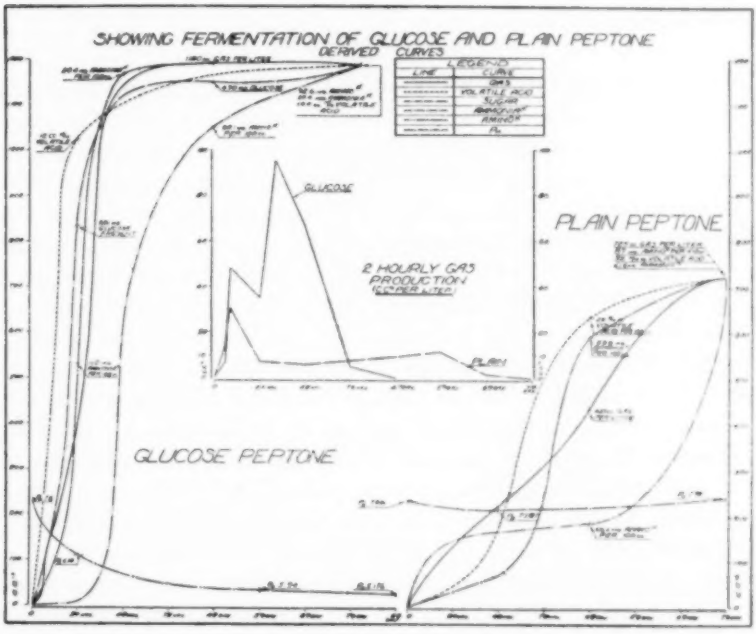


CHART 2. CHANGES IN PEPTON MEDIA DUE TO THE GROWTH OF B. BOTULINUS

these curves practically coincide. The amino acid accumulation is apparently not dependent on the other chemical changes. In the plain pepton culture the gas and ammonia production curves tend to parallel each other. This behavior suggests a close relationship between the two. It is conceivable that the amino acids are deaminized and that the remaining carbon fraction is subsequently degraded by beta-oxidation with the liberation of

CO_2. In the glucose culture the amino acid curve takes an independent course.

The results of the two experimental series are so similar, that the one reported in detail may be considered as a prototype of the growth of *B. botulinus* in plain and glucose pepton solutions. The likeness is especially significant when it is recalled that *B. botulinus* Type A in Parke-Davis pepton was compared with Type B in Difco pepton.

Experimental series 3

Van Ermengem (1897) procured the strongest *B. botulinus* toxin in a medium containing 2 per cent glucose. There are some indications that the American strains produce stronger toxins in the absence of glucose. For example Shippen states, "In dextrose mediums the acidity of the reaction supposedly is not favorable to toxin production. Since growth without acidity occurs in the presence of lactose, this substance was used in place of dextrose." Dubovsky and Meyer discontinued the use of glucose media in their isolation work on account of the acidity, which inhibited the toxin production in soil cultures. Glucose has been found to favor slightly the toxin production of *B. botulinus* Type A strains 38 and 97 in a well buffered medium (Dozier, Wagner and Meyer). Since the metabolic activities of *B. botulinus* in 2 per cent solution are completely changed by the presence of glucose it was thought that its effect on toxin formation could be more readily observed in this medium than in one of higher biological value. Accordingly toxin tests were added to the regular chemical determinations. These experiments were in every way similar to those already reported. Guinea pigs were used for testing the progressive toxicity of the cultures.

In the first series Difco pepton was the medium and an actively growing thirty-six hour old culture of *B. botulinus* strain 6 (Type B) was used for seeding. The data are shown in table 3. After a lapse of forty-eight hours unusually vigorous growth took place in both cultures as judged by turbidity, gas production and the appearance of the microscopic slide preparations.

A loss of amino acids was noted in the sample removed on the twenty-fourth hour. The enzymic machinery for splitting off amino acids was probably more active after that time. At the forty-eighth hour, in spite of the ammonia which accumulated in the sugar-free culture, the amino acids increased. The changes

TABLE 3

Toxin production of B. botulinus in pepton and glucose-pepton solutions

2 PER CENT DIFCO PEPTON					AGE OF CULTURE	2 PER CENT DIFCO PEPTON + 1 PER CENT GLUCOSE					
Volatile acid N/10	Ammonia N	Amino N	pH	Toxin M.L.D.		Toxin M.L.D.	pH	Amino N	Ammonia N	Volatile acid N/10	Sugar
Type B, strain 6											
cc.	*mgm.*	*mgm.*		*cc.*		*cc.*		*mgm.*	*mgm.*	*cc.*	*per cent*
1.4	2.8	58.2	8.0 C	–	0 hours	–	8.0 C	56.0	2.8	2.0	1.14
–	–	34.7	7.9 C	1.0	24 hours	1	7.3 C	49.0	–	–	–
6.6	9.9	65.0	7.6 C	1.0	48 hours	1	5.0 C	51.4	4.2	7.8	0.90
35.6	40.6	91.2	7.53 E	0.5	72 hours	1	5.02 E	64.0	7.0	–	0.63
53.7	67.2	93.3	7.54 E	0.05	96 hours	1	4.89 E	69.9	–	9.5	–
–	–	–	–	0.005	118 hours	1	–	–	–	–	–
71.5	109.0	83.5	7.47 E	0.0005	142 hours	1	4.85 E	76.5	9.8	9.8	0.40
Type B, strain 53											
2 PER CENT PARKE-DAVIS PEPTON						2 PER CENT PARKE-DAVIS PEPTON + 1 PER CENT GLUCOSE					
3.6	4.2	63.2	7.8 C	–	0 hours	–	7.8 C	63.2	4.2	5.2	1.60
–	39.2	73.9	7.4 C	1.0	20 hours	Neg.	5.0 C	50.7	7.0	–	1.56
37.2	46.9	83.7	7.54 E	0.5	44 hours	1	4.88 E	62.5	–	10.8	0.75
39.2	70.0	95.0	7.42 E	–	68 hours	–	4.70 E	47.7	–	13.0	0.75
–	–	–	–	0.005	92 hours	Neg.	–	–	7.0	–	–
50.6	76.0	76.2	7.18 E	0.005	7 days	–	4.65 E	74.2	8.5	–	0.60

in reaction and the sugar consumption of 740 mgm. per 100 cc. were similar to those previously reported. The toxin in the plain pepton culture increased steadily; on the one-hundred-forty-second hour 0.0005 cc. of the centrifuged broth killed a guinea-pig in less than four days. In the culture, which contained glucose, little or no toxin was formed.

Parke-Davis pepton was inoculated in the second series with a *B. botulinus* Type B, strain 53. The chemical analyses indicate that on account of the favorable reaction the culture became active somewhat sooner than that in the first series. The determinations which extended over a period of seven days show, however, that the chemical changes taking place are greater in the glucose-free medium of the first than of the second series. More sugar disappeared from the second culture than from the first and a correspondingly greater H-ion concentration developed. The toxin formed in the culture of the second series was not as strong as that of the first.

The presence of glucose inhibited the toxin production of Type B *B. botulinus* in 2 per cent Difco and 2 per cent Parke-Davis pepton solutions. The general appearance of the cultures indicated that more organisms were present in the glucose than in the glucose-free cultures; the toxin was therefore not dependent upon the number of organisms. The acidity, which resulted from the metabolism of the carbohydrate probably did not affect the toxin directly. It is recalled that Dickson (1915) found a potent toxin in the cultures of *B. botulinus* at an acidity of 3.2 per cent to phenolphthalein and workers in this laboratory have repeatedly demonstrated the presence of toxin at a pH of 4.2. Accepting the hypothesis discussed and developed by Dozier, Wagner and Meyer, that the bacterial cell is the matrix of the poison and that the toxin is formed upon the autolysis of the organism, it is reasonable to assume that any agent, which interferes with autolysis, may influence the production of toxins. Flexner, Sturges and Rettger (1922) and others have studied the autolysis of bacteria. While little is definitely known concerning the enzymes which bring about this destruction Bradley has shown that proteolytic and autolytic enzymes, are not identical and that the latter are more easily inhibited by changes in reaction. The presence of glucose and readily available nitrogen gives little impetus for the production of nitrogen disintegrating enzymes. The acidity developed so quickly in the poorly buffered media that there was little time during which a favorable reaction existed for autolytic enzymes to act. Near

the critical reaction for autolytic enzymes growth probably occurred without toxin production. According to Dernby and Allander *B. tetani* extends its growth over a broad pH range; while its toxin production is narrowed to a pH of 6.2 to 8.5. However, in a more recent paper Dernby and Walbum present evidence which indicates that the toxin production of *B. diphtheriae* is dependent on the presence of albumoses in the medium and on the reversal to a definite H-ion concentration. This aspect of the problem will be discussed later, more in detail.

Supplementary tests have shown that the toxin production of Type A, strains 38 and 97 is not inhibited by the presence of glucose to the same degree as that of Type B cultures. Additional comparative tests were unfortunately impossible on account of the fact that some months later strains 53 and 6 as well as other B strains had changed to biotypes, which were nontoxic in pepton solutions. Variations and even loss of the toxin producing ability have been observed in Type C cultures of *B. botulinus* (Wagner). The enzymes or chemical factors engaged in the generation of toxins of the B and C Types are undoubtedly more readily affected by their environment than those of the comparatively stable Type A strains.

Experimental series 4

The addition of gelatin to a peptic-digest broth increases its biological value for *B. botulinus*. In fact media containing 10 per cent gelatin are successfully used in this laboratory for the mass production of spores. This protein was originally added to the broth in order to buffer the excessive production of acids which apparently inhibited spore production. In this connection it is recalled that the value of gelatin in media was attributed to (1) changes in the viscosity and consistency (North), (2) increase in buffer substance and (3) presence of nutritive elements (Worth). The recently published studies of Graham-Smith favor the last named property. He found by extensive plate counts that the total number of staphylococci grown in an 8 per cent gelatin solution and in a meat extract broth was less than the number grown in an 8 per cent gelatin-meat extract medium alone. It

seemed of interest to verify this conception by analyzing the metabolic processes of *B. botulinus* grown in pepton-gelatin medium with and without glucose.

Gelatin to make approximately a 10 per cent concentration was added to a 2 per cent Difco pepton solution. The Type B,

TABLE 4

Growth of B. botulinus on pepton gelatin and glucose-pepton gelatin

2 PER CENT DIFCO PEPTON + 10 PER CENT GELATIN					TIME	2 PER CENT DIFCO PEPTON + 10 PER CENT GELATIN, 1 PER CENT GLUCOSE					
Volatile acid N/10	Ammonia N	Amino N	pH	Gas per liter		Gas per liter	pH	Amino N	Ammonia N	Volatile acid N/10	Sugar
Strain 53 (series A)											
cc.	*mgm.*	*mgm.*	"E"	*cc.*		*cc.*	"E"	*mgm.*	*mgm.*	*cc.*	*per cent*
2.2	6.0	93.6	6.1	0	0 hours	0	6.0	84.4	6.0	2.2	1.04
6.5	—	—	6.19	5	24 hours	134	5.98	—	—	7.5	0.91
17.8	39.0	143.0	6.77	73	48 hours	1032	5.41	170.0	31.0	18.4	0.59
28.8	—	156.8	6.68	116	72 hours	1785	5.27	170.6	—	24.6	0.40
—	—	—	6.43	191	96 hours	2500	—	—	—	—	—
—	—	156.6	—	226	116 hours	2800	5.19	185.0	—	—	0.02
141.2	326.0	357.8	6.77	762	11 days	—	5.57	220.0	56.2	29.0	0
Strain 38 (series B)											
1.2	0	113.0	8.27	0	0 hours	0	8.27	101.0	0	2.6	0.96
—	0	108.0	—	11	24 hours	4	—	99.3	0	—	0.88
4.6	18.2	123.0	7.9	73	66 hours	770	7.24	139.0	28.0	12.2	0.71
—	—	—	—	160	74 hours	1185	—	—	—	—	—
86.6	176.4	398.0	7.6	580	140 hours	2446	5.3	341.6	38.0	41.0	0
143.0	350.0	420.0	7.0	822	9 days	2446	5.3	347.0	47.0	43.0	—

strain 53 was used for Series A and the Type A, strain 38 for Series B. The data are summarized in table 4.

It is evident from table 4 (series A) that the greatest chemical changes took place between the twenty-fourth and forty-eighth hours of incubation. It was expected that after the exhaustion of the glucose the nitrogenous constituents would be more vigorously attacked. The medium contained 1038 mgm. of nitrogen per 100 cc. and only 186 mgm. or 16.9 per cent was

broken down by the organisms to amino acids and ammonia in eleven days. Although the pH reverted from 5.19 on the one-hundred-sixteenth hour to 5.57 on the eleventh day it is reasonable to assume from previously recorded observation that the growth of *B. botulinus* was unfavorably influenced by the reaction and some of the volatile acids and failed to regain its original vigor. The evolution of gas was very slow in the absence of glucose. At the conclusion of the experiment only 762 cc. per liter, or approximately the same amount as was formed by the organism in sugar-free pepton, were recovered. The retention of the carbon in the form of volatile acids and its unavailability for CO_2 production possibly account for the moderate gas production in a relatively rich medium. The proteolytic activity of the organisms increased the concentration of the amino acids and ammonia so that after eleven days incubation 66 per cent of the total nitrogen was present in the form of these split-products.

In Series B the reaction of pH 8.27 delayed the activity of the organisms; it was not until after the sixty-sixth hour of incubation, when the reactions became pH 7.9 and pH 7.2 respectively that vigorous chemical changes took place. After the initial lag period the growth of the organisms caused strikingly similar changes in the media of the two experimental series.

The acidities developed in the glucose-gelatin-pepton cultures indicate that the gelatin added little if any buffer value to the 2 per cent Difco pepton solutions. This is confirmed by titration experiments in which it was found that 2 per cent gelatin solutions had the low buffer index of 0.4 per cent normal. Further reference to this fact will be found in the sixth experimental series.

Chart 3 presents the data from Series B in the form of derived curves. It can be seen that in the glucose culture, the greatest accumulation of gas, amino acid, ammonia and volatile acid occurred during the same time interval. From table 4 it is evident that no more gas was formed after the sugar was exhausted. The complete metabolism of the sugar did not entirely paralyze the activity of the culture. The continued formation

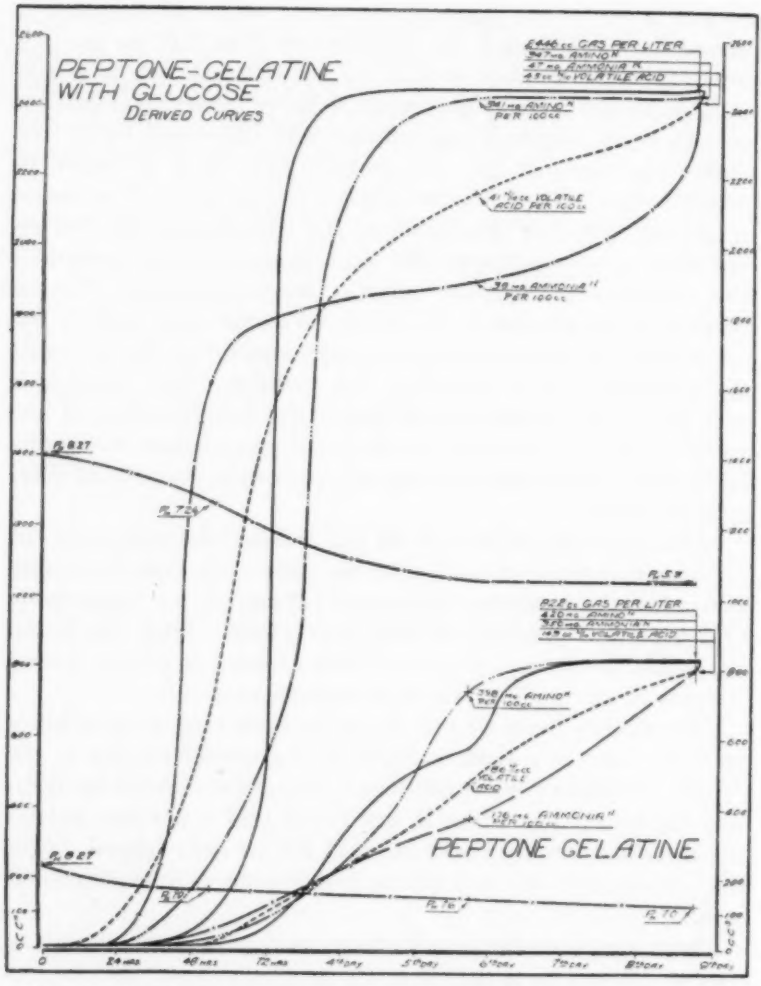


CHART 3. THE ADDITION OF GELATIN TO PEPTON MEDIUM INCREASED THE METABOLIC ACTIVITY OF B. BOTULINUS

of ammonia and volatile acid indicated clearly that gas production in glucose culture was not dependent on the formation of these products. Furthermore the curves indicate that in the absence

of glucose the gas evolution and the nitrogen breakdown are intimately related.

A comparison of the activities of *B. botulinus* in a 2 per cent pepton and in a 10 per cent gelatin-2 per cent pepton solutions reveals that a larger amount of glucose is metabolized by the organisms in the gelatin medium and that the evolution of gas is correspondingly increased. The ammonia accumulation, while representing about the same percentage increase, amounts to much more in the gelatin-glucose culture. If taken as an index of intercellular metabolism as suggested by Kendall it signifies a much heavier growth than in the 2 per cent pepton-glucose cultures (series 2). Moreover the formation of amino acid and ammonia which occurred in the gelatin-pepton cultures is indicative of greater proteolytic activity. Experiments, which are not detailed in this communication, have shown that *B. botulinus* is unable to grow in plain watery non-hydrolyzed gelatin solutions. However, it is definitely proven that this protein incorporated into a simple pepton medium furnishes additional nutritive material. Little evidence was secured to support the contention that gelatin increases the true buffer value of the medium.

Experimental series 5

In the preceding series *B. botulinus* was studied on media, which, aside from a liberal amount of amino acid, contained also a fair proportion of polypeptides and albumoses. It appeared to be of interest to determine the biological activities of this anaerobe in a nutritive solution which was biuret-free and yet rich in animo acids. Such a medium was secured by using the commercially available aminoids. Aside from carrying out biochemical analyses it was important to answer the following questions: (1) Are amino acids inhibitive or toxic for *B. botulinus*, and (2) does the bacterium produce toxins in a biuret-free medium? Concerning the first question it is recalled that Burrows and Neymann noted a toxic or inhibitive influence of concentrated amino acid solutions on young tissue cells. More recently McLeod and Wyon report a similar unfavorable effect

of special amino acids on *B. proteus* and *B. pyocyaneus*. These observations have as far as is known not been extended to anaerobes, although it is definitely proven that *B. botulinus* can develop in a 20 per cent solution of Difco pepton (Miss Bristol in this laboratory). The second question will be considered in subsequent paragraphs. It need merely be emphasized that according to Robinson and Rettger (1917), Bunker, Dernby and Walbum and others, toxin production of *B. diphtheriae* is slight or entirely absent in media which lack proteoses and peptons. The findings reported in this paper leave no doubt that *B. botulinus* is capable of toxin formation even in the absence of these split-products.

It furthermore seemed advisable to include determinations which would detect losses in the nitrogen content of the medium since *B. botulinus* is a soil organism and through processes of nitrification may cause such a depletion.

A 5 per cent solution of aminoids (a preparation sold by the Arlington Chemical Company, and probably a cereal digest product) was prepared in distilled water with 0.5 per cent NaCl. The solution was adjusted to the optimum reaction; 65 cc. were placed in large test tubes, vaseline stratified and sterilized. Three drops of a twenty-four hour culture of *B. botulinus* Type A, strain 38 were used for inoculating the tubes. At the end of twenty-four hours the cultures showed vigorous growth. On the ninety-first hour chemical tests were made, and the results are reported in table 5.

The data presented in table 5 indicate that in the course of ninety-one hours the amino acid content of the medium decreased 139.8 mgm. An accumulation of 210 mgm. of ammonia accounted for the amino nitrogen loss. It signifies that the organisms metabolized a portion of the unidentified nitrogen. An estimation of the exact amount of the total of 156 mgm. utilized would entail the determination of the nitrogen resident in the bacterial bodies. A small amount of the original 156 mgm. of undetermined nitrogen was in the form of amino acid which was not accounted for by the Van Slyke determination, the rest was probably in the form of simple peptids.

Considerable gas and volatile acids were formed; the reaction changed very slightly. Toxins were formed though the exact titer was not determined; in other tests *B. botulinus* has produced

TABLE 5

Growth of B. botulinus Type A on aminoids solution

	5 PER CENT AMINOIDS		
	Control	91 hours culture	Loss or gain
Total nitrogen, mgm	655.0	—	—
Amino acid N, mgm	454.5	315.0	−139.8
Ammonia N, mgm	44.3	210.5	166.2
pH, E	7.06	7.28	
Volatile acid N/10, cc	4.0	67.2	+63.2
Toxin, 0.1 cc. killed guinea-pig in 21 hours			

	2 PER CENT AMINOIDS			2 PER CENT AMINOIDS + 1 PER CENT GLUCOSE		
	Control	4 days	10 days	Control	4 days	10 days
Total nitrogen, mgm	283.5	288.0	290.0	275.0	273.0	271.6
Amino N, mgm	197.0	158.0	125.0	184.7	171.3	169.2
Ammonia N, mgm	18.5	72.8	84.0	18.0	34.0	37.3
pH, C	7.8	7.4	7.4	7.8	5.5	5.5
Gas per liter, cc	0	680.0	685.0	0	1010.0	1010.0
Glucose, per cent	—	—	—	1.7	—	1.10

TABLE 6

Growth of anaerobes in a 5 per cent aminoids solution

	VOLATILE ACID N/10	AMMONIA N
	cc.	*mgm.*
B. tetani, 91 hours old cultures	42.0	144.0
B. histolyticus, 91 hours old cultures	97.8	173.0
B. sporogenes, 91 hours old cultures	—	224.0
B. botulinus, 91 hours old cultures	67.2	210.0

in a four day old aminoids culture a toxin, which in a dilution of 1:50,000 was fatal to mice.

Parallel experiments with *B. tetani*, *B. sporogenes* and *B. histolyticus* were made on the same 5 per cent aminoids solution (table 6). The increases in ammonia and volatile acids in the ninety-one hour cultures were indicative of vigorous growth.

B. histolyticus produced little gas compared with the other organisms; there was however a conspicuously larger accumulation of volatile acid and a lower pH (6.76).

In a second series *B. botulinus* was studied in a 2 per cent solution of aminoids, with and without glucose. The gas was carefully collected and samples of the cultures were analyzed at regular intervals. The analytical data are shown in table 5.

The amount of gas (table 5) and its rate of evolution from the glucose-aminoids medium differed in no way from that reported for the glucose-pepton cultures. The volume of gas recovered from the plain aminoids substratum was somewhat larger than that ordinarily collected from plain pepton media. Six hundred mgm. of sugar were used by the organisms during ten days fermentation. An excess of 1100 mgm. was still present. The reaction in the sugar-free culture remained almost constant, while in the glucose-containing culture it dropped to pH 5.5.

Enzymes capable of liquefying carbolized gelatin solution were present in the seventy-two hour old glucose and in the plain aminoids cultures of *B. botulinus*. The stimulation of enzymes is apparently independent of the complex nitrogenous compounds of the medium.

Total nitrogen determinations show that the nitrogen content of the media remained almost constant; differences of 6.5 mgm. and 3.4 mgm. are considered insignificant. The reaction of the media (pH 7.4 to 5.5) favored the retention of ammonia. It was only after cultures of *B. tuberculosis* became distinctly alkaline that Bosworth, Elkins and Blanchard observed noteworthy losses of ammonia. Since *B. botulinus* is prone to reduce an alkaline medium to a lower pH there is little chance for such extensive nitrogen losses as those reported by the above workers for the tubercle bacillus. Gas analysis of *B. botulinus* cultures show the presence of minute amounts of NO_2 (Miss Anderson in this laboratory). The losses which occur through oxidation are probably too small to be detected by the usual Kjeldahl determinations.

Amino acids in concentration as high as 454 mgm. of nitrogen per 100 cc. exerted apparently no inhibitive or toxic effect upon the organism but rather seemed to stimulate growth.

Experimental series 6

Witte's pepton always has been considered the standard for bacteriological work. Since the war, however, its use has been restricted and its composition probably changed (Wilcox, 1922) so that no agreement exists among workers as to which of the many available peptons are the most satisfactory. Wolf and Harris (1917), for example, used the Cole and Onslow digest solution; Wilcox (1921) found Berna pepton highly satisfactory; Kendall failed to state, in a recent publication, the type he selected; Foster (1921) in metabolism studies of streptococcus employed Parke-Davis pepton; Dernby and Walbum were able to continue the use of Witte's and De Bord working with *B. botulinus* and other organisms used Difco pepton.

Through the studies of Robinson and Rettger (1918), Davis, Bunker and others it is well known that the biologic value and chemical composition of the commercial peptones vary greatly. Although they are each said to support growth of most bacteria little is known concerning their value in a medium, which is used for the growth and the toxin production of *B. botulinus*. Furthermore no comparative study of the nature of the nitrogen components has been published.

The most important inquiries concerning the value of peptones for *B. botulinus* seemed to be: (1) which are the most suitable for growth; (2) which support the greatest toxin production; (3) what are the differences in the nitrogen constituents and buffering properties; (4) what chemical changes take place in the course of the development of the organism on the various peptones; (5) and is there any chemical evidence that peptic-digest, which has been successfully used in this laboratory, is superior to commercial peptones for cultivating *B. botulinus*.

Peptones have been "valued" for the most part by incorporating the test pepton into a standard medium and then comparing the ability to support growth and toxin production of such organisms as *B. diphtheriae* and *B. tetani*. There is no necessity for introducing the variables present in broth, in appraising the biologic value of peptones for *B. botulinus* because this organism is able to grow on watery solution of peptones.

Five of the most commonly used commercial peptones, a biuret-free product, aminoids, gelatin and peptic-digest prepared in this laboratory were selected for the comparative study. With the exception of the peptic-digest, which was made according to the formula given by Stickel and Meyer, 2 per cent solutions of the test material were prepared with 0.5 per cent NaCl and adjusted with NaOH to the optimum reaction. These solutions were distributed unfiltered into Blake bottles, vaseline stratified and autoclaved one-half hour. It is of interest to note the differences in appearance of these media after cooling. The color varied from a straw yellow to a brown in the following order Berna, Witte, Parke-Davis, Difco, Fairchild, aminoids and peptic-digest. Berna, Witte and peptic-digest solutions contained a considerable amount of flocculent precipitate while only a small amount of insoluble material was detected in the others.

Two sets of these solutions were used for the chemical analysis of the peptones and two sets were inoculated with 1 cc. of a heated *B. botulinus* meat culture rich in spores. The growth, the toxin production and the extent of the chemical changes caused by the organism were studied.

The nitrogen constituent of these media is divided into proteose, non-protein, unidentified, amino acid and ammonia fractions. Determinations were made of the total nitrogen and of the non-protein nitrogen. The difference between the two fractions represents the nitrogen available in the form of complex molecules. Gelatin contains native protein while the peptones as digestion products furnished only proteose or albumoses. In recent experiments Hiller and Van Slyke found that "tungstic acid was distinguished for the relative completeness with which it precipitated protein intermediary substances without precipitating amino acids." The tungstic acid filtrates however gave a biuret reaction and suggest that there were peptid groups present. Kendall (1922) called the non-protein nitrogen which was not identified as amino acid or ammonia, "polypeptid nitrogen." Since such a fraction contains not only the peptid nitrogen but also amino acids not determined by the Van Slyke

TABLE 7

Comparison of various bacteriological peptones

	2 PER CENT SOLUTIONS								
	Witte*	Parke-Davis	Difco	Berna	Aminoids	Fairchild	Peptic-digest†	Diluted peptic-digest	Gelatin
Total nitrogen, mgm	267.0	278.0	289.0	239.4	283.0	245.0	672.0	267.0	277.0
Proteose nitrogen, mgm	175.3	78.5	21.0	151.4	0	0	277.0	110.5	235.0‡
Nonprotein nitrogen, mgm	91.7	199.5	268.0	88.0	283.0	244.0	395.0	156.5	42.0
Amino nitrogen, mgm	30.3	76.3	70.6	44.0	197.0	105.0	219.0	86.5	21.0
Unidentified nitrogen, mgm	59.9	118.6	196.2	41.6	67.6	126.8	153.6	61.2	21.0
Ammonia nitrogen, mgm	1.5	4.6	1.2	4.4	18.4	12.2	22.4	8.8	0.0
Buffer Index per cent N, colorimetric	1.6	2.2	1.5	1.5	2.2	1.82	5.95	2.32	0.4
Buffer index, per cent N, electrometric	1.62	2.2	1.48	1.36	6.2	1.72	5.95	2.32	0.16
Ash, per cent air dried material	1.5	2.25	4.12	6.75	8.5	3.75	—	—	—
P_2O_5, per cent air dried material	0.42	0.47	0.72	0.18	1.7	0.61	—	—	Trace

* Prewar pepton.

† Peptic-digest made according to Stickel and Meyer.

‡ Protein nitrogen.

method, creatin, creatinin, uric acid and traces of other nitrogenous compounds that are known to be present in small amounts this faction is here designated as the "unidentified nitrogen" of the medium. The results from the determinations are to be found in Table 7.

The nitrogen content of the seven commercial products averaged about 13 per cent of the air dried material; the peptic-digest was much richer in nitrogen. For comparison the analytical figures secured from the sample of peptic-digest investigated were reduced to the nitrogen content of Witte's pepton. These figures are shown in a separate column of table 7.

It is generally assumed that the commercial peptones consist of a mixture of protein degradation products. It can be seen that Witte, Berna and peptic-digest have, roughly speaking, half of their nitrogen in complex nitrogen molecules. Aminoids and Fairchild pepton lack such a fraction and Parke-Davis and Difco peptones possess only a small amount of nitrogen which is precipitated by tungstic acid. The statement made by Davis that the American peptones lack albumose and proteose compounds which are present in Witte's pepton is supported by these observations. The solutions rich in complex nitrogen compounds, with the exception of peptic-digest are relatively poor in amino nitrogen. When diluted to the same concentration as Witte's the amino acid of peptic-digest compares favorably with the amounts present in Parke-Davis and Difco peptones. Aminoids and Fairchild pepton are rich in amino acids. The "unidentified nitrogen fraction" is abundant in Difco, and present in fair amounts in Fairchild and Parke-Davis pepton. Comparatively little ammonia was found in any of the solutions.

The buffer indices of the test solutions have been determined by colorimetric and electrometric titrations. The results from the latter are presented in curves (chart 4).

An agreement between the two types of titrations was attained with the exception of those for aminoids. The electrometric titration curve (chart 4) indicates that between pH 7.6 and 8.0 the aminoids solution was able to accommodate a large amount of alkali. This made it most difficult to read the exact end point with an indicator.

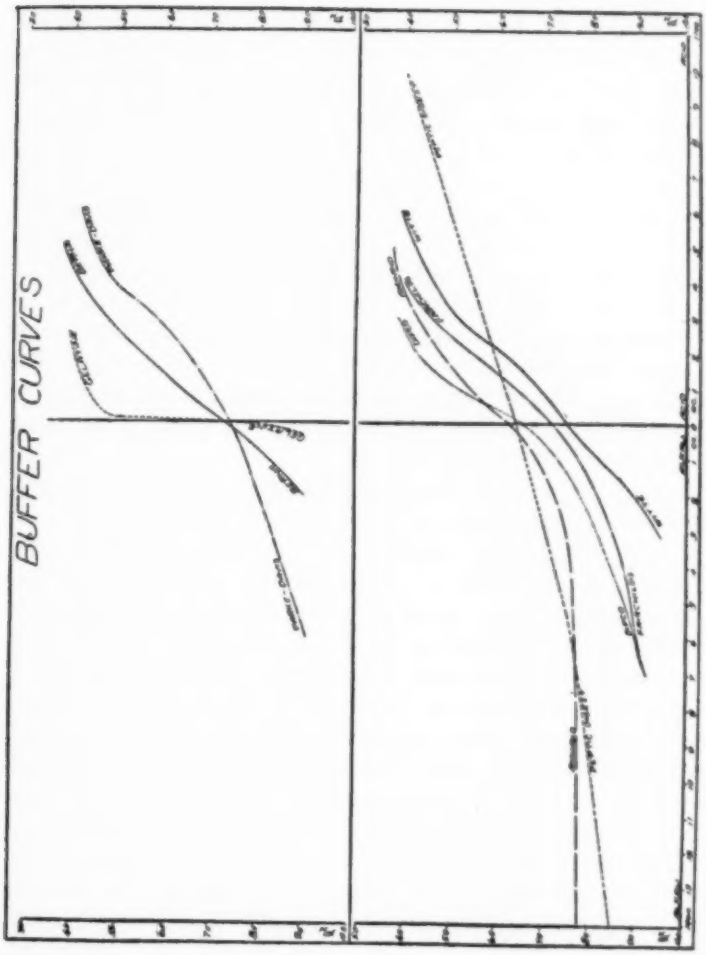


CHART 4. THE CHANGES IN REACTION OF MEDIA CAUSED BY THE ADDITION OF STANDARD ACID AND ALKALI

Peptic-digest with dibasic phosphate, as ordinarily prepared in the laboratory, possesses considerable reserve alkalinity which is most desirable; when diluted to the same nitrogen content as Witte's pepton solution its buffer index is still slightly higher than that of any of the other solutions. It was of interest to discover that unhydrolyzed gelatin has comparatively little ability to resist changes in reaction. The buffer values between pH 8.0 and 5.0 recorded for Difco, Witte and aminoids solutions are similar to those reported by Bronfenbrenner, De Bord and Orr. The Fairchild pepton used by them had a higher buffer index than that employed in these experiments. Considerable variation exists between different lots of the same brands of peptones, for example lot no. 11,052 of Difco pepton made up with redistilled water had a pH of 7.2 while lot no. 11,572 prepared with water from the same container had a pH of 6.5. It is not unlikely that had the buffer values been determined, variations as great as those reported for Difco pepton would have been found for Fairchild pepton.

One gram of each of the peptones was ashed and weighed. Witte's and Berna which are similar in their nitrogen fractions showed very dissimilar amounts of ash. The amounts and composition of the ashes from Berna and aminoids were apparently quite similar. Only a few of the more conspicuous differences in the composition of the various ashes will be mentioned. A yellow iron oxide was present after burning Difco pepton. The large amount of iron gives rise to the black iron sulphide, which is quite definite in Difco pepton cultures of proteolytic anaerobes. Hall (1924) also found that the greatest blackening occurred in Difco media. In the ash from the Fairchild pepton, a copper compound was detected. It probably came from the alcohol which had been dehydrated by the copper sulphate method and used for the precipitation of the pepton.

The phosphate content of the peptones was estimated by uranium acetate titrations. There is an exact agreement in the amount of P_2O_5 found by Davis in Witte's pepton and that reported in this experiment. Difco contained more phosphate than any of the other peptones. Aminoids however was much

TABLE 8

Changes produced in pepton solution by B. botulinus Type A, strain 97, upon six days incubation

	WITTE	PARKE-DAVIS	DIFCO	BERNA	AMINOIDS	FAIRCHILD	PEPTIC-DIGEST	GELATIN
Amino nitrogen:								
Initial, mgm	30.3	76.3	70.6	44.0	197.0	105.0	219.0	21.0
After growth, mgm	102.7	79.8	98.4	103.7	116.1	117.0	262.0	19.0
Change produced by organism, mgm	72.4	3.5	27.8	59.7	−80.9	12.0	43.0	−2.0
Ammonia nitrogen:								
Initial, mgm	1.5	4.6	1.2	4.4	18.4	12.2	22.4	0.0
After growth, mgm	42.3	95.2	48.0	70.9	64.2	49.0	206.8	4.0
Produced by organism, mgm	40.8	90.6	46.8	66.5	45.8	36.8	184.8	4.0
Reaction:								
Initial, pH	7.6	7.6	7.0	7.4	6.5	7.0	6.5	7.2
After growth, pH	6.9	6.9	6.6	6.7	6.6	6.8	7.0	6.7
Buffer value:								
Initial, per cent N	1.6	2.2	1.5	1.5	2.2	1.8	5.95	0.4
After growth, per cent N	4.0	6.4	6.4	4.9	4.6	3.75	12.3	0.6
Growth:								
Macroscopic	Fair	Excellent	Excellent	Good	Fair	Fair	Excellent	Poor
Microscopic examination	Spores	No spores	High per cent spores	Few spores	Spores	Few spores	High per cent spores	No spores
Toxin—after six days incubation, 1 cc. fatal to mouse in dilution of	1:10,000	1:1,000	1:5,000	1:100	1:50,000	1:5,000	1:100,000	1:1

the richest in that constituent. Peptic-digest is always deficient in phosphate, and 0.2 per cent is therefore added in the course of its preparation.

After six days incubation at 37°C. the media which had been inoculated were analyzed. With the exception of the gelatin solution the growth of the organisms caused an evolution of gas and marked turbidity. The comparative macroscopic and microscopic appearance of the cultures are recorded in table 8.

The amino acid determinations indicate that in Witte's and Berna solutions with relatively low amino acid nitrogen content a considerable accumulation of the split-products took place. In a medium (Parke-Davis and Fairchild) rich in amino acids, approximately the same concentration was maintained throughout the entire growth period. A definite loss of amino nitrogen was noted in the aminoids solution.

A marked accumulation of ammonia occurred in all the cultures. The organism produced the most profound chemical changes in the Parke-Davis pepton as judged by the ammonia production; in fact proportionally more was formed in this pepton than in the peptic-digest. The changes in reaction were slight and probably had little effect on the growth of the organisms or its ferments.

B. botulinus caused in every pepton solution a definite increase in the ability of the medium to resist changes in the reaction on the addition of acid or alkali. Brown (1921) noted that the growth of *B. coli* increased the buffer index of plain bouillon; in the presence of glucose this ability was slight, which naturally suggested that the increase in buffer value was due to the protein metabolism. It has been generally assumed that the amino acids played a rôle in the buffering of a medium through their terminal amino and carboxyl groups. Recently Robertson has entirely disproved this theory. He found that hydrolized gelatin had less ability to resist change in pH than unhydrolized, which he attributed to a loss of $-CONH-$ groups. Likewise peptic-digest an enzymic product rich in amino acids was found by Stickel and Meyer to be relatively poor in buffer substance. The deficiency is corrected by the addition of dibasic phosphate.

The apparent increase in buffer material in the 6 day old cultures is probably due to the accumulation of alkaline and acid products which are held in the medium in the form of weak acid and alkali salts. Provided this explanation is correct the high buffer index developed in Parke-Davis and peptic-digest is accounted for by the large accumulation of ammonia. In the Difco pepton, as has already been mentioned, more volatile acids and a higher H-ion concentration developed than in any of the other peptones (see also Discussion, Experimental series 2).

Peptic-digest and Difco cultures of *B. botulinus* gave positive indol reactions with Ehrlich's reagents; when, however, they were tested by the Goré method, which is more specific, no reactions were obtained.

In every pepton solution *B. botulinus* toxin was produced. The variations are not due to the nitrogen constituents of the media. Aminoids and Witte media are very dissimilar and yet both yielded a strong toxin. The low toxic titer developed in the Berna culture was unexpected since this pepton proved highly satisfactory for the commercial manufacture of tetanus toxin (Wilcox). When Berna pepton was incorporated in a veal-broth it repeatedly furnished highly potent toxins. Berna pepton evidently lacks some specific substance supplied by the veal-broth. The weak toxin found in the gelatin solution undoubtedly developed from the germination of the spores, since there was little or no evidence of active growth.

Experimental series 7

The phenomenon of putrefaction of meat has long been the subject of chemical research. Early experiments were of a general nature and dealt primarily with the recognition of putrefactive products and their isolation. Later Tissier and Martley and Rettger determined the changes occurring in meat due to the growth of supposedly pure cultures. Muriel Roberston and subsequently Holman, Heller and others introduced the cooked meat mash medium into the cultural study of anaerobic or facultative anaerobic bacteria. Wolf and Harris have published interesting papers on the biochemical behavior of *B. sporogenes*,

B. histolyticus and *B. welchii* in this substratum. In this connection it is recalled that Th. Smith (1890) and later Tarozzi, Noguchi and others employed tissue broth cultures for the isolation of delicately growing microörganisms. As far as is known, no biochemical studies have been made on such media. Although beef heart mash media are not entirely satisfactory for quantitative biochemical studies (Wagner, Dozier and Meyer) it nevertheless appeared advisable to report on a number of studies which have been made with *B. botulinus* grown in this substratum. The abundance of waste products, which accumulate in a beef heart mash culture of *B. botulinus* furnish a suitable source for their separation and identification. The general composition of the medium has been frequently changed in this laboratory, and in order that there may be no misunderstanding the preparation is given in detail: The fat and the fasciae are removed and the meat ground. A given amount is mixed with twice its weight of water and heated slowly in a double boiler to 95°; this temperature is maintained for one hour. After cooling, the broth is freed from floating fat by skimming and is separated from the meat particles, which are reground. Later both fractions are recombined, the reaction adjusted and stabilized by heating. The finished medium is distributed in flasks in the desired amounts. It is customary to autoclave for one hour the first day and one-half hour the second. Between the two sterilizations the flasks are kept at 37°C.

In the course of various experiments conducted in the laboratory the question was frequently asked, when does *B. botulinus* produce the maximum amount of toxin and when does deterioration take place at 37°C.? Thus far each group of workers has chosen the incubation time for toxin production more or less arbitrarily. At 28°C. an incubation period of one month or even longer has been practiced, while at 37°C. Hall (1924) found highly potent toxins to be present after the second or third day of incubation. Bengtson (1921) in her article on the standardization of the botulinus antitoxin found the maximum amount of toxin in beef heart cultures on the tenth to the fourteenth day. In a former paper the course of the progressive toxin

production in a veal infusion-peptic digest broth has been reported. Although in comparison with the poison of *B. welchii* and *B. diphtheriae* the botulinus toxin is very stable it is reasonable to suspect that it may deteriorate when kept at 37°C. These considerations led to an experimental series, in which the toxin production in the meat mash was studied simultaneously with the biochemical changes.

Approximately 1750 cc. of sterilized meat medium prepared as previously described, were placed in 2-liter Pyrex flasks and sterilized. Shortly before the inoculation, the medium was reheated to expel CO_2 and covered with a one-half inch layer of petrolatum. A mercury cup seal as used by Madsen for the cultivation of *B. tetani* protected the culture from exposure to the air. One flask was inoculated with 10 cc. of a forty-eight-hour-old beef heart culture of Type A, strain 38, and the other with a similar culture of Type B, strain 40. Samples for analysis were removed with a siphon. Suitable dilutions of the centrifuged cultures were made in saline and injected intraperitoneally into guinea-pigs.

Both cultures after the twelfth hour of incubation showed evidences of growth. The meat particles appeared first swollen, then discolored and finally by the eighth day their contour was completely lost. On the eleventh day whitish crystalline deposits (perhaps tyrosine) were noticed in the pasty mass of disintegrated meat. The liberation of gas was most active around the forty-eighth hour. There was no perceptible macroscopic difference between the Type A and Type B cultures at any time during the entire period of observation. Microscopically, both cultures revealed a predominance of spores. The data of this series are presented in table 9.

The medium contained approximately 1320 mgm. of nitrogen per 100 cc.; about 4 per cent was present in form of amino acids and ammonia. At the end of sixteen days of incubation the A and the B Types converted 60 and 50 per cent respectively of the total nitrogen into soluble amino acid and ammonia compounds. In the A culture the amino acids increased most rapidly between the thirty-sixth to forty-eighth hour; the rate of accumulation

then decreased although some amino acids were formed throughout the entire experimental period. The rate of ammonia formation was similar, but the decline was more gradual than in the

TABLE 9

Growth and toxin production of B. botulinus on beef heart mash

TIME	AMINO NITROGEN	AMMONIA NITROGEN	AMINO AND AMMONIA NITROGEN FORMED BY ORGANISM	TOXIN M.L.D.	VOLATILE ACID N/10	pH
			Type A, strain 38			
	mgm.	*mgm.*	*mgm.*	*cc.*	*cc.*	"C"
0 hours	30.9	28.0	0	0	4.0	6.8
6 hours	39.0	25.5	0	—	6.6	6.6
12 hours	34.5	38.9	14.5	1.0	—	6.8
24 hours	56.0	49.3	46.4	1.0	8.5	6.8
36 hours	60.8	83.2	85.1	0.1	15.5	6.6
48 hours	116.7	124.1	181.9	0.01	21.5	6.9
72 hours	188.5	204.0	333.6	0.001	48.0	6.9
120 hours	242.6	278.0	461.7	0.0001	85.0	—
8 days	373.0	391.8	706.8	0.0001	98.0	6.9
11 days	474.4	431.8	847.3	0.00001	141.0	—
16 days	433.5	496.0	870.0	0.001	157.0	6.7
			Type B, strain 40			
0 hours	30.0	23.0	0.0	0	4.0	6.8
6 hours	33.0	25.5	0.0	—	4.5	6.5
12 hours	31.7	25.8	13.5	—	6.0	6.8
24 hours	43.4	49.9	35.5	—	12.8	6.7
36 hours	60.7	69.7	77.4	1.0	16.5	6.7
48 hours	70.0	90.1	107.1	0.1	21.5	6.7
72 hours	188.5	207.0	342.5	0.01	44.5	—
120 hours	—	—	—	0.001	—	6.7
8 days	357.0	346.0	650.0	0.001	93.0	—
11 days	335.0	402.0	687.0	0.0001	—	—
16 days	383.0	421.0	771.0	0.001	114.0	6.7
			Control			
7 days	31.1	28.5			4.1	6.7

amino acid production. The proteolytic activity of the mass culture developed slowly in the B culture. The peak was reached on the fifth day and then dropped gradually to a low level. The

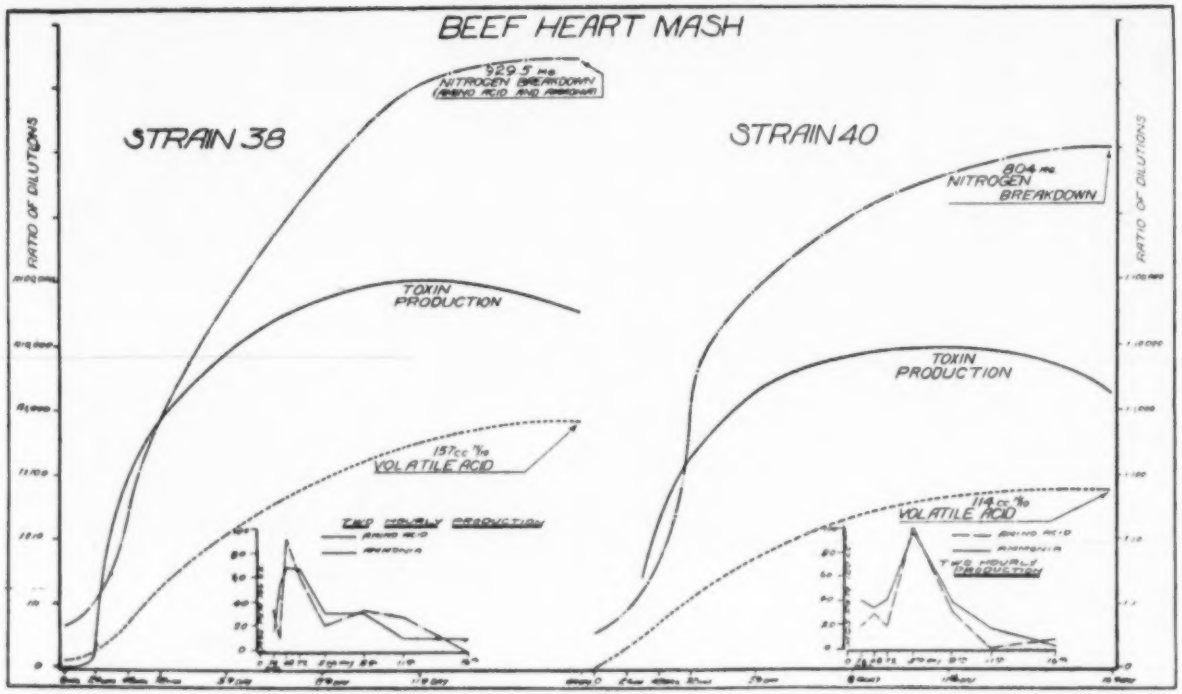


CHART 5. THE NITROGEN DISINTEGRATION AND TOXIN PRODUCTION CAUSED BY TWO STRAINS OF B. BOTULINUS

hourly production curves presented in chart 5 illustrate this statement. Volatile acids developed in both cultures, but in spite of the large amounts demonstrated the reaction of the medium fluctuated very little. In fact the H-ion concentration remained almost constant.

The sum of the amino and ammonia nitrogen is used as an index of the proteolytic activities of a culture and is termed the "nitrogen breakdown" shown in chart 5.

A glance at the curves suggests that the toxin formation is closely connected with the increase in nitrogenous split-products. The potency rapidly progressed between the thirty-sixth and the one hundred twentieth hours in the Type A and between the forty-eighth and the one hundred twentieth hours in the Type B culture. Both reached their maximum toxicity on the eleventh day and showed a slight diminution in strength on the sixteenth day. These observations convey the impression that the toxin production of *B. botulinus* in meat mash is in some way related to the proteolytic changes, which take place in the culture. The process of toxin formation is probably intimately connected with the proteolytic enzymes formed by the organism, but it is not as simple as the available data would imply. In this connection it is recalled that *B. sporogenes* or non-toxic cultures of *B. botulinus* induce a protein breakdown, which is similar to that of *B. botulinus* although no botulinus-like toxin is produced.

The findings fail to support the contention of Knorr, who in quoting Geiger states that the Type B produces a stronger toxin than the Type A strains. However, it must be emphasized that strain 40 generally grows less actively than some other members of the B group studied in this laboratory. It is therefore not unlikely that the difference in the potency of the toxin must be attributed to the reduced vigor of growth. This point will be discussed later. The data of this experimental series have been confirmed in a number of similar tests and have prompted Dubovsky and Meyer to select an incubation period of ten days for the study of their field cultures.

Experimental series 8

Nothing definite is known concerning the oxygen requirements of *B. botulinus*. As a rule, growth is entirely absent in liquid media, which contain no meat and are not stratified with petrolatum. Since it is customary in this laboratory to cultivate *B. botulinus* under varying conditions it appeared advisable to study the products of metabolism, which formed in an inoculated beef heart mash medium exposed to varying oxygen tensions.

For this experiment, 20 bottles each containing 150 cc. of beef heart medium were used; 9 were inoculated with 0.5 cc. of a vegetative culture of strain 38, Type A, and 9 with a similar culture of strain 40, Type B; 2 sterile flasks served as controls. In each set, 3 bottles were closed with cotton plugs, the media of 3 others were covered with a half inch layer of petrolatum and 3 were evacuated and sealed. They were incubated at 37°C. Chemical analysis were made on the contents of one bottle of each group on the fifth, tenth and fifteenth day of incubation. The results of the tests conducted on the tenth day are presented in chart 6; the progressive protein disintegration of the medium under the influence of the bacterium is shown in table 10.

Growth was initiated at about the same time in all of the flasks. By the tenth day considerable amounts of amino acids and ammonia accumulated. In the Type A cultures the exclusion of oxygen (chart 6) favored the formation of ammonia. The Type B culture covered with petrolatum was less active for reasons which have not been determined. In the sealed cultures the reaction remained slightly acid probably due to the retention of CO_2 and H_2S. In the open flasks the escape of these gaseous waste products rendered the medium slightly alkaline. The total amounts of volatile acids formed in the three ten days old Type A cultures were practically the same: 95 to 120 cc. N/10 acid per 100 cc. of medium. Unfortunately the types of acids have not been determined.

The data presented in table 10 indicate that the exclusion of oxygen favors the proteolytic activities of *B. botulinus*. Furthermore they support the contention of Omelianski that the com-

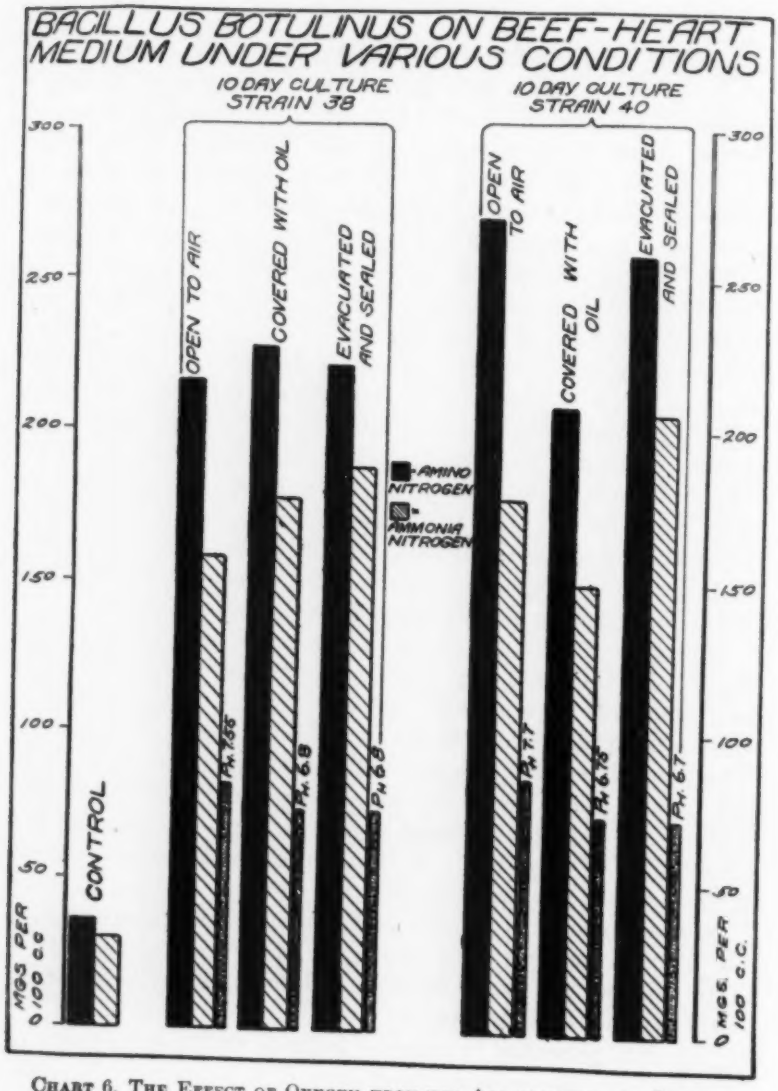


CHART 6. THE EFFECT OF OXYGEN UPON THE ACCUMULATION OF GROWTH METABOLITES

position of the medium determines the degree of anaerobiosis necessary for the growth of an obligate anaerobe. Invariably, excellent growth and toxin production are noted in broth containing meat particles. Whether the action of the meat is merely mechanical as suggested by Douglas, Flemming and Colebrook or whether it serves as a reducing agent as experimentally proven by Gates and Olitsky deserves further investigation.

Experimental series 9

In the preceding experiments the amount of volatile acid developed in the cultures of *B. botulinus* has been determined. It appeared of interest to identify the various acids. Meat

TABLE 10

Per cent of total nitrogen recovered as amino acid and ammonia

CULTURES	TIME	OPEN TO THE AIR	COVERED WITH OIL	EXHAUSTED AND SEALED
		per cent	*per cent*	*per cent*
Type A, strain 38	5 days	19.0	20.0	21.5
	10 days	28.7	31.4	32.9
	15 days	35.0	40.0	42.0
Type B, strain 40	5 days	16.0	17.0	24.2
	10 days	34.5	28.0	36.9

mash cultures were chosen and in order to elucidate the mechanism of the acid formation, simultaneous determinations of the amino acids, the ammonia and other metabolic waste products were made. Seven cc. of a thirty-six-hour-old meat culture Type A (strain 38) were used to seed 1750 cc. of beef heart medium; the evolution of the gas was measured and samples of the cultures were removed at regular intervals. The data of this experimental series are presented in table 11.

It is evident from the figures presented in table 11 that the gas production was vigorous; by the twenty-eighth hour 465 cc. per 1000 cc. of medium had accumulated; a second period of active gas evolution occurred after the fortieth hour. The heavy sampling of the culture on the seventh day, which was necessary

for the acid identification tests, probably interfered with the subsequent gas measurements.

In the course of ten days the non-protein nitrogen of the medium was increased from 101 to 1050 mgm.; 787 mgm. of this fraction were identified as amino acids and ammonia. There is little need for comment upon the accumulation of these soluble nitrogen compounds as they were very similar to those formed by *B. botulinus* in the preceding experiments. Determinations on similar meat cultures indicated that 60 mgm. of nitrogen were

TABLE II

Growth of B. botulinus strain 38 on beef heart mash

TIME	GAS PER LITER	AMINO NITROGEN	AMMONIA NITROGEN	NON-PROTEIN NITROGEN	VOLATILE ACID N/10	pH
	cc.	*mgm.*	*mgm.*	*mgm.*	*cc.*	"C"
0 hours	0	32.0	18.7	101	1.6	6.7
4 hours	46	—	—	—	—	—
16 hours	251	—	—	—	—	—
21 hours	—	67.9	44.2	—	10.8	6.68 E
28 hours	461	—	—	—	—	—
40 hours	512	111.5	117.0	—	33.6	6.9
52 hours	792	—	—	—	—	—
64 hours	1055	—	—	—	—	—
72 hours	—	250.0	127.0	—	75.0	7.2
88 hours	1150	—	—	—	—	—
112 hours	1210	—	—	—	—	—
120 hours	—	357.0	261.0	—	118.0	7.2
136 hours	1260	—	—	—	—	—
7 days	—	343.0	340.0	966	141.0	—
10 days	—	374.0	413.0	1050	158.2	7.4
30 days	—	523.0	438.0	—	217.0	—

present in the form of creatinin while only traces of urea and uric acid were detected. Volatile alkaline amines in small amounts were also determined. The largest part of the 263 mgm. of unidentified soluble nitrogen was resident in the peptid nitrogen compounds and in amino acids, as for example proline, which is not accounted for in the Van Slyke determinations. As has been previously stated by Wagner, Dozier and Meyer, the non-protein nitrogen determinations show that a greater nitrogen cleavage took place in the medium than was indicated

by either or both the amino acid and ammonia figures. The reaction of the culture became more alkaline as growth proceeded. In a control culture in which no arrangement was provided for the release of the gas, the pH dropped from 7.4 to 6.5.

Volatile acids. The volatile acid accumulated fairly regularly throughout the experimental period, and 217 cc. of N/10 acid were recovered after thirty days incubation of the culture.

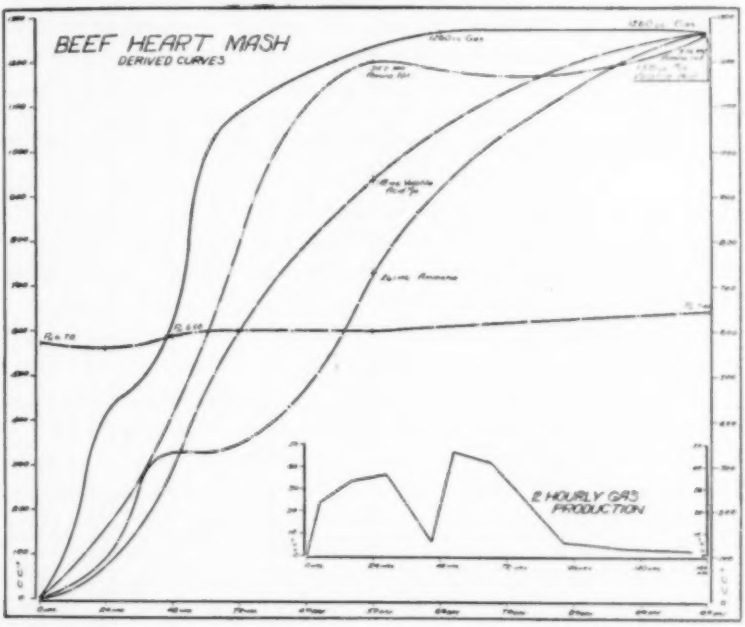


CHART 7. THE DISINTEGRATION OF MEAT MEDIUM CAUSED BY B. BOTULINUS

The derived curves (chart 7) indicate that the volatile acids resulted from the nitrogen disintegration of the medium; their accumulation was more regular than that of the ammonia. Long chain acid radicals could be oxidized during the growth of the organism without disturbing the $-COOH-$ group. *B. botulinus* is probably not able to carry the oxidizing process further than acetic acid since tests for formic acid repeatedly

yielded negative results. The accumulation of volatile acids would in such a case be more regular than that of the ammonia. Deaminizing procedures in the medium and excretory waste material from the bacterial bodies both undoubtedly increase

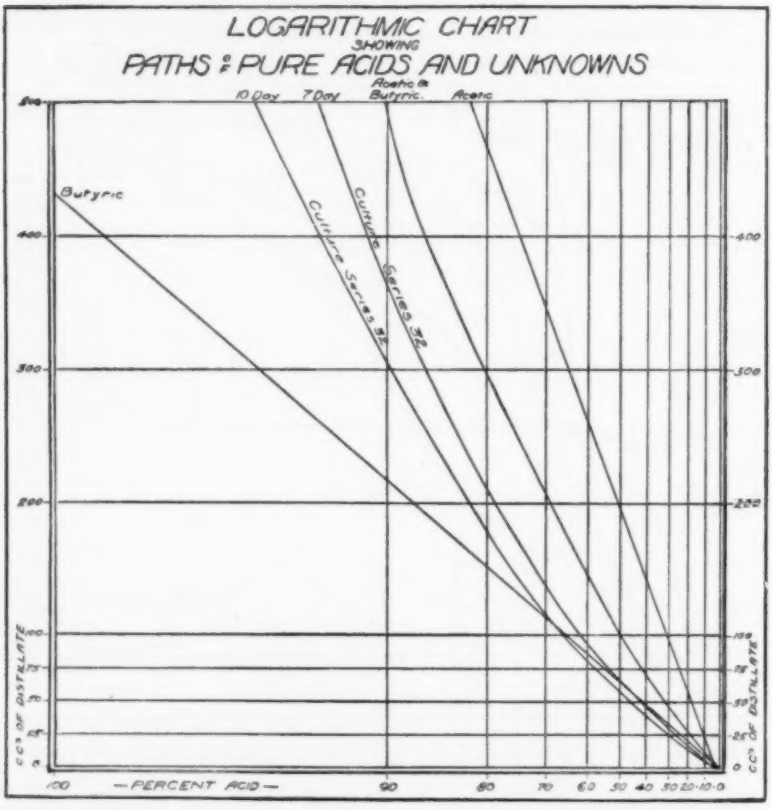


CHART 8. THE STEAM DISTILLATION OF KNOWN AND UNKNOWN ACID MIXTURES

the ammonia nitrogen of the culture. For the identification of the volatile acids three modifications of the Duclaux methods[3] have been used.

[3] In order to avoid any misunderstanding the methods which have been tried and those which were finally chosen, are briefly reviewed.

1. Steam distillation. Pure acids are distilled with steam according to the Dyer technique. The results from the titration of the 100 cc. fractions of distillate are calculated as percentages of the total amount of acid known to be present. The rate of volatilization of acetic and butyric and also of a mixture of the two are shown in chart 8. By a comparison of the results from the steam distillation of an unknown acid mixture with those of known acids an approximate idea of the nature of the acids present is secured. Color tests serve excellently for identifying individual volatile acids but give little information when applied to mixtures of closely related acids.

2. Fractional steam distillation. The methods of Wolf and Telfer for fractional distillation were found impractical and their use was discontinued.

3. Duclaux method. Solutions of known acids are made up to a volume of 110 cc. and distilled in 10 cc. fractions, these are titrated and the results calculated as percentages of the distillate in 100 cc. The sequence of the resulting numbers is characteristic for each acid. Most satisfactory results are secured by this method and it was adopted for the determination of acids in fermenting mixtures.

The direct distillation of the media was inadvisable on account of frothing and the subsequent changing of the organic material. The cultures were therefore distilled with steam until 1500 cc. of the distillate had been collected. The volatile acids were titrated, an excess of alkali added, evaporated on the water bath to 75 cc.; then the acids were liberated with H_2SO_4 and the mixture was distilled by the Duclaux technique. The titration sequence of the known acids in the preceding experiments agreed so closely with the results of Duclaux, that the tables prepared from the distillation of mixtures of acids by him were used for the interpretation of the data collected from this experiment. To facilitate further the identification of the acids the first five 10-cc. fractions and the last five 10-cc. fractions of the distillate after titration were combined and redistilled. After the acid solution was fractionally distilled for the second time positive color tests on the separated portions of distillate were obtained for valeric, butyric and acetic acid.

4. Gravimetric determinations. Additional information concerning the identity of the volatile acids was secured by preparing their sodium salts. After titrating the volatile acids the salts were dried and weighed. The weight of the sodium which neutralized the acids was easily computed. This amount of sodium was calculated as if it were in combination with the various volatile acid radicals which the preceding distillations indicated to be present in the culture. The weight of the theo-

TABLE 12

Volatile acid produced by B. botulinus in 10 day meat culture

		WHOLE CULTURE	MIXTURE* BUTYRIC, 7 VALERIC, 3 ACETIC, 2	FIRST FRACTION OF CULTURE	MIXTURE* 1 VALERIC, 1 BUTYRIC, 1	SECOND FRACTION OF CULTURE	MIXTURE* BUTYRIC, 2 ACETIC 1
Percentage of volatile acid in fraction of distillate	10 cc	23.6	—	23.7	24.1	13.9	—
	20 cc	36.8	—	42.8	43.3	27.1	—
	30 cc	51.4	48.9	57.2	58.5	38.4	39.5
	40 cc	61.6	60.6	68.2	70.5	48.5	50.8
	50 cc	71.0	70.1	77.5	79.5	58.0	60.7
	60 cc	78.3	78.1	84.0	86.5	66.8	69.8
	70 cc	84.5	84.7	89.4	91.5	75.0	78.0
	80 cc	90.0	90.5	93.5	—	83.9	85.6
	90 cc	95.2	95.5	97.0	—	90.2	—
	100 cc	100.0	100.0	100.0	—	100.0	—
Volatile acid as sodium salt—found, grams		—		0.7375	—	0.3285	
Calculated as sodium salts in proportion found by titrations, grams				0.747		0.325	

* Duclaux tables.

retical amount of volatile acid salt should agree with what is recovered by drying and weighing. This sodium in combination with volatile acid radicals in the proportion indicated by the titration figures should give a weight of salt corresponding to the amount actually found.

An approximate idea of the nature of the acids present in a seven- or a ten-day-old beef heart mash culture of *B. botulinus* was obtained by comparing the figures of the steam distillate of 50 cc. with those indicated in the distillation curves of known

acids (chart 8). These data were controlled by the Duclaux method. The computations are presented in table 12.

The data indicate that a mixture of valeric, butyric and acetic acid in the proportion of 3:7:2 was present in the cultures. Subsequent fractional distillation furnished conclusive evidence that the interpretation of the figures is correct. The weight of the calculated sodium salt agreed closely with the amount actually found. In 100 cc. of a ten-day-old culture there were present 80 cc. of N/10 butyric, 35 cc. of N/10 valeric and 232 cc. N/10 acetic acid. Determinations were also made on thirty-day-old cultures; the amount of the volatile acids had increased but no difference, either in the kind or in the proportion, of the acid were found. Schüble found only butyric acid in glucose-liver broth cultures of *B. botulinus*. This result is probably due to the methods employed by him, which were not sufficiently delicate to distinguish between the closely related members of the volatile acid series.

Fixed acids. Determinations were made for fixed acids by extracting for seventy-two hours in a continuous liquid ether extractor 40 cc. of the acidified culture; after removing the ether, the watery extract of the acids was made up to 500 cc. and boiled over a direct flame to remove the volatile acids. The remaining 75 cc. were filtered, and washed and an aliquot portion was titrated with N/10 barium hydroxide. In a thirty-day culture 54.4 N/10 cc. of fixed acids were present. The barium salt solution was evaporated and dried, then converted into barium sulphate, dried and weighed. There was 0.23 gram which would be equivalent to 50.5 cc. N/10 lactic acid. This result suggests that the fixed acid in a *B. botulinus* beef heart culture is probably lactic acid.

Experimental series 10

The extensive work on the isolation and distribution of *B. botulinus* carried on in this laboratory offered an excellent opportunity to secure a number of strains for biochemical study. While very different results from individual metabolism studies are to be found no comparisons have been made of the activity

of organisms obtained from different sources, grown and analyzed under identical conditions.

The organisms isolated for less than a year before this chemical study was made and those which were tranplanted only during the course of their purification are designated as "new strains"; those which were received from other laboratories and repeatedly purified by a number of passages are recorded as "old strains."

It seemed worth while to compare the growth activity of a number of the old laboratory strains with some of the recently isolated ones and also to compare the biochemical behavior of *B. botulinus* with that of a number of commonly studied anaerobes.

Twenty-five strains of *B. botulinus* were used in these experiments. Their purity and virulences were repeatedly tested and will therefore not enter into the discussion. Two strains each of the *vibrion septique*, *B. sporogenes*, *B. histolyticus* and *B. tetani* were included in the series.

The media chosen for this study were meat mash and milk. The first possesses a high biological value while the second offers an excellent opportunity for making visual comparisons. Van Ermengem's organism failed to show any metabolic activity in milk, while Von Hibler reported that *B. botulinus* precipitated the casein of milk with subsequent peptonization. Such differences of opinion concerning the behavior of *B. botulinus* on milk have persisted. The historical aspect of the subject is considered more in detail in connection with a subsequent experiment.

Duplicate Blake bottles containing 150 cc. of beef heart medium or milk were inoculated with 0.5 cc. of twenty-four to thirty-six hour old cultures of the chosen organisms. Sterile rubber stoppers bearing bent glass tubes were placed over the cotton plugs and held in place by Imperial Cement. Anaerobiosis was secured by exhaustion.

The cultures were incubated for relatively long intervals in order to secure striking differences in the chemical behavior of the organisms. The cultures were observed from time to time; growth was apparently regular in the duplicate meat flasks. The *B. botulinus* cultures, with the exception of strain 4, as well

as the *B. sporogenes* and *B. tetani* cultures after fifteen days of incubation reduced the meat particles so that they appeared as a dark colored pasty mass in the bottom of the bottles. Determinations were made on the fifteenth or nineteenth day and again on the thirtieth or sixtieth day.

Ordinarily the milk became coagulated after thirty-six hours, though often it took several days. The soft curd was soon rent with gas bubbles which usually rose to the surface of the liquid, the whey gradually being squeezed out. In a few days the curd appeared as a hard lump either floating on the surface or at the bottom of the flask. A gradual disintegration then took place and in most cases by the fourteenth day only a small amount of solid material remained. Variations however were noticed; for example, strain 24 caused only a soft coagulation which seemed to melt away, while strain 32 gave rise to a granular curd. The milk used in this experiment was heated for three consecutive days in an Arnold sterilizer. Caramelization occurred in some of the bottles which probably accounted for the difference in the color of the whey and may have been partially responsible for the nature of the curd.

Tetanus cultures caused no apparent change in the milk until the fourteenth day. The milk was however eventually digested; in thirty days the proteolytic activities had proceeded to the same degree as in the *B. botulinus* cultures. *Vibrion septique* caused the formation of a soft clot (eight days) but there was little evidence of further chemical activity. *B. sporogenes* and *B. histolyticus* both produced profound changes in the milk curd, similar to those observed in the majority of the *B. botulinus* cultures.

The presentation of all the data from the chemical analyses would be a needless repetition. The results have therefore been averaged and are summarized in table 13.

Nitrogen determinations on a number of different bottles of the meat medium indicated that in spite of the precautions which were taken the meat had not been accurately distributed (940 to 825 mgm.). The average nitrogen content was 870 mgm. per 100 cc., a medium less rich than that which was used in the

preceding experiments. A part of the apparent growth variability in the meat cultures (table 13) can be attributed to the composition of the medium. When averages were taken of the amounts of the amino acids, ammonia, and volatile acids present in the fourteen to eighteen-day-old meat cultures, it was noted that the "new strains" showed slightly greater chemical acitivity than the "old strains." The variations were decidedly less in the milk. The averages indicate very little difference between the "old" and "new strains."

The analytical data of 5 strains of *B. botulinus*, selected at random, are compared with those of *vibrion septique*, *B. sporogenes* and *B. tetani* cultures. To facilitate the comparison the amino acid and ammonia formation are shown graphically in chart 9.

TABLE 13

Meat and milk cultures of B. botulinus

	AGE OF CULTURES	NUMBER OF CULTURES	AMINO NITROGEN PRODUCTION (AVERAGE)	AMMONIA PRODUCTION (AVERAGE)	VOLATILE ACID PRODUCTION (AVERAGE N/10 ACID)
			mgm.	*mgm.*	*cc.*
Meat:					
"Old strains"....	14–18 days	14	157.0 (94–272)	104.2 (75–187)	45.7 (25–85)
"New strains"...	14–18 days	7	162.0 (96–200)	128.4 (75–180)	46.3 (31–83)
Milk:					
"Old strains"....	14–15 days	6	182.6 (145–228)	135.2 (112–160)	54.6 (43–70)
"New strains"..	10–12 days	11	146.2 (75–186)	109.4 (39–172)	42.2 (22–60)
"New strains"...	21 days	6	200.3 (181–231)	139.9 (101–175)	51.0 (35–68)

Five per cent of the nitrogen of the medium was present as amino acids and ammonia. In the *vibrion septique* culture after 18 days incubation a slight increase of the soluble nitrogen compounds occurred. Similar changes in meat media were observed by Wolf who found that after one-hundred two hours 4.8 per cent of the total nitrogen was transformed into amino acid and ammonia. In these experiments the red color of the meat mash was intensified by the growth of the *vibrion septique;* the other organisms caused the meat particles of the medium to

become dark colored and disintegrated. The reaction of pH 6.4 in the *vibrion septique* culture was further evidence of differences in the chemical activity on the part of the organism. The *B. sporogenes* cultures were quite active and provoked slightly larger accumulations of amino acids and ammonia after an eighteen days incubation period than did *B. botulinus*. The pH was greater than 7.0. In the tetanus cultures the chemical

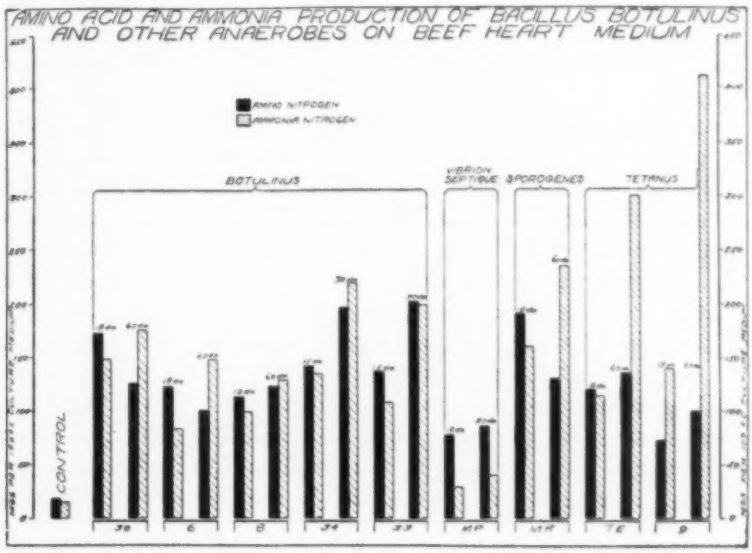


CHART 9. AMINO ACID AND AMMONIA PRODUCTION OF B. BOTULINUS AND OTHER ANAEROBES ON BEEF HEART MEDIUM

changes developed slowly, but after prolonged incubation greater amounts of ammonia nitrogen were present than in any of the cultures thus far examined.

Experimental series 11

Th. Smith (1890) and recently Wolf (1922) called attention to the presence of muscle sugar in ordinary bouillon. Smith (1893), with the methods at his disposal, considered gas production by aerobes, and by obligate anaerobes a certain test for sugar

fermentation. Hall (1921) found considerable quantities of gas were produced by *B. tetani*, *B. welchii* and *vibrion septique*, but not by *B. botulinus*, *B. sporogenes* or *B. bifermentans* cultured in sugar-free broth in special marble-sealed sidearm fermentation tubes. For the preparation of sugar-free broth Randall and Hall advise the use of *B. welchii* as a more vigorous sugar fermenter than *B. coli* or *B. saccharolyte*. Hall (1921) considered an increase in the H-ion concentration as the best indication of fermentation. In media containing less than an excess of fermentable carbohydrate proteolysis would supervene only after the exhaustion of the sugar, and the first signs of a reversal toward alkalinity could therefore be used as an indication of the point at which resterilization should be undertaken.

Studies on the metabolism of *B. welchii* which have been made in this laboratory have failed to reveal any evidence of a secondary acid reversion in reaction in either beef-infusion or double strength veal-infusion. All the available data indicate that a reversion in reaction in muscle free cultures of *B. welchii* is the best criterion of exhaustion of the sugar of the medium.

One phase of the fermentation question which Smith (1893) dealing with *B. diphtheriae* appreciated, but which many later workers seem to have ignored, is the possible depletion of other constituents than glucose of the medium by preliminary fermentation with organisms such as *B. coli*, *B. saccharolyte* and *B. welchii*.

No one who has worked with cultures of *B. welchii* can have failed to marvel at the profound changes which growth of the organism can produce. Aside from the very evident utilization of the fermentable carbohydrates, the colloidal consistency of the media after a few hours growth is suggestive evidence of biochemical changes at least as important, and perhaps even more fundamental, from the standpoint of the biologic value of the media for a second growth of bacteria.

The difference in media depleting power of different species of organisms is well illustrated by Graham-Smith's work. His results also show clearly the selective nutritional requirements of several organisms, regarding which he writes:

The growth of any of these organisms in the medium seems to remove most of the food for that species as well as a portion of the food substance used by other species, since in no case was the growth of the added species nearly so considerable in extent as in its primary cultures.

Preliminary to some studies on the glycolytic enzymes of *B. botulinus* it was deemed advisable to study the metabolism of the anaerobe in a medium which had been deprived of its carbohydrates by a preceding fermentation with *B. welchii*. Double strength veal-infusion—1 per cent Difco pepton, pH 7.2, was autoclaved at 115 pounds pressure for one-half hour. One-half of the lot was then distributed in 200 cc. Blake bottles in 100 cc. portions, and vaseline stratified. The second half was fermented with *B. welchii* for twenty-one hours,[4] autoclaved for one hour, filtered through paper, the reaction adjusted to pH 7.2, and distributed in the same manner as the first half. It should be noted that filtration did not entirely remove the *B. welchii* organisms. The bottles were incubated for forty-eight hours as a test for sterility and heated for twenty minutes in flowing steam just prior to inoculation. Each flask received 1 cc. of a spore suspension (*B. botulinus* strain 19) heated for one hour at 80°C. The bottles were incubated at 37°C. and one of each series served for the determinations at the intervals listed in table 14. The initiation of growth was far from uniform in the different bottles, and those which appeared to have the most representative growth were chosen. It was the unanimous decision of the several workers concerned that the separate bottle method introduces a higher percentage of error than the single container method, involving the complicated sampling procedure

[4] It is deemed important to describe here briefly the nitrogenous metabolism which take place in a veal-infusion-pepton broth in the course of the *B. welchii* fermentation. The only biochemical change detected during the first fifteen hours of incubation is a slight increase in the H-ion concentration. By the twenty-first hour the organisms attacked the nitrogen fraction of the medium causing an increase of 13 mgm. of amino acid and 27.2 mgm. of ammonia nitrogen, while the reaction became slightly alkaline. In a beef heart peptic-digest medium the changes brought about by *B. welchii* at the end of eighteen hours incubation are even more striking. The amino acid content increased from 136.9 to 188 mgm. and the ammonia from 17 mgm. to 57.8 mgm. per 100 cc.

previously described. The only advantage of the multiple bottle method, convenience excepted, is the avoidance of possible

TABLE 14

Growth of B. botulinus strain 19 on double strength veal infusion + 1 per cent Difco pepton

TIME	TOTAL N	AMINO N	AMMONIA N	VOLATILE ACID N/10	pH	ORGANISMS PER CUBIC CENTIMETER
			"A" medium unfermented			
	mgm.	*mgm.*	*mgm.*	*cc.*	"C"	
0 hours	259.8	63.6	22.0	6.8	7.2	22
12 hours	–	59.0	17.6	–	7.1	22
18 hours	–	68.0	16.5	7.8	7.1	30
24 hours	–	59.6	21.3	–	7.1	19,000
30 hours	–	52.1	23.6	–	6.9	77,500,000
36 hours	–	58.9	33.6	–	6.6	7,900,000
42 hours	–	70.0	32.5	12.4	6.6	152,500,000
48 hours	246.4	61.1	35.8	–	6.6	80,000,000
54 hours	–	68.0	28.0	–	6.6	80,500,000
60 hours	–	72.0	43.7	13.6	6.6	25,000,000
72 hours	249.0	63.5	47.6	–	6.8	154,500,000
84 hours	–	118.0	47.0	16.6	6.8	43,800,000
96 hours	248.2	70.0	52.0	–	6.6	11,000,000
5 days	–	70.9	52.5	17.4	–	–
7 days	246.0	63.0	57.7	20.6	6.7	–
			"B" medium fermented with *B. welchii*			
0 hours	294.0	72.8	30.0	13.6	7.2	4
12 hours	–	84.0	27.2	–	7.1	5
18 hours	–	84.0	27.2	14.8	7.1	60
24 hours	–	71.0	34.8	–	7.1	14,000
30 hours	–	74.8	38.2	–	7.0	25,125,000
36 hours	–	79.8	33.5	–	7.1	654,000
42 hours	–	81.6	39.8	19.2	7.0	20,150,000
48 hours	280.0	81.1	41.5	–	6.9	5,750,000
54 hours	–	95.2	36.2	–	6.9	31,400,000
60 hours	–	99.6	57.8	24.6	6.9	2,150,000
72 hours	282.0	92.8	70.5	–	6.9	22,875,000
84 hours	–	83.9	69.0	26.2	6.9	4,317,500
96 hours	275.2	63.7	72.6	–	6.9	18,560,000
5 days	–	48.2	50.9	32.8	–	–
7 days	285.0	69.7	82.5	34.2	6.9	–

effects on growth processes of agitation of the media incident to sampling. This decision is in harmony with the conclusion of

Sears, who discarded separate bottles after a comparison of the two methods.

Plate counts made immediately after inoculation, and after twelve and eighteen hours respectively, indicated relatively small inoculums, which offered a finer test of the biologic value of the two media. Proliferation was slow in both the fermented and unfermented media, and the loss in biologic value from the fermentation with *B. welchii* is very evident from the averages of plate counts. The greater growth in the unfermented bottles is also indicated by the greater depression of the pH than in the fermented bottles, which may of course be attributable to the stimulating presence of muscle sugar.

The nitrogen content of the original veal-infusion was extremely low, and is undoubtedly to be explained by the fact that "bob" veal was used in its preparation. The amino acid changes were relatively slight in both media, but in spite of the smaller number of organisms in the fermented medium ammonia accumulated in slightly greater amounts; this was true also of the volatile acids. While these chemical findings suggest some carbohydrate sparing action, it seems hardly possible to attribute all the higher biologic value of the unfermented medium to its sugar content.

Experimental series 12

Double strength veal-infusion 1 per cent Difco pepton was fermented with an active strain of *B. welchii* for twenty-one hours. The medium was heated, filtered, placed in 2-liter Erlenmeyer flasks and sterilized. To one flask was added enough 50 per cent glucose to make a 2 per cent concentration, and to another in addition to the glucose enough sterile dibasic phosphate solution to make a 1 per cent concentration. The flasks were arranged in the 37°C. water bath, and anaerobiosis established, after which each received 20 cc. of a heated spore suspension of *B. botulinus* strain 19.

Total nitrogen determinations revealed a nitrogen content of 573 to 628 mgm. per 100 cc., an amount more than double that of the medium used for experimental series 11. The latter, as

TABLE 15

Growth of B. botulinus on double strength veal infusion + 1 per cent Difco pepton

TIME	TOTAL N	NON-PROTEIN N	AMINO N	AMMONIA N	VOLATILE ACID N/10	TITRATABLE ACID N/1	pH	SUGAR	GAS PER LITER	ORGANISMS PER CUBIC CENTIMETER	MICROSCOPIC APPEARANCE	GELATINASE (PER CENT GELATINE LIQUEFACTION)
Fermented veal-infusion pepton												
	mgm.	*mgm.*	*mgm.*	*mgm.*	*cc.*	*cc.*	"E"	*per cent*	*cc.*			
0 hours	628.0	364	137.0	53.4	20.0	0.55	7.23	–	0	1,250	100% Gram –	0
12 hours	–	–	131.0	56.0	–	–	7.21	–	116	176,500	100% Gram –	0
18 hours	–	–	146.0	65.6	–	0.8	7.21	–	170	5,000,000	90% Gram – : 10% Gram +	0
24 hours	–	–	129.0	72.8	–	0.9	7.24	–	267	219,500,000	88% Gram + : 12% Gram –	75
30 hours	–	–	125.0	53.2	50.0	1.1	7.25	–	423	252,000,000	–	90
36 hours	–	–	126.0	106.5	–	1.15	7.31	–	–	274,000,000	–	90
42 hours	–	–	120.6	103.6	–	1.45	–	–	–	22,100,000	98% Gram – : 1% Gram + : 1% spores	90
48 hours	–	476	121.0	81.2	49.8	–	7.2	–	493	50,300,000	98% Gram – : 1% Gram + : 1% spores	75
54 hours	–	–	121.0	78.4	–	1.35	7.15	–	–	39,450,000	–	90
60 hours	–	–	121.2	110.3	–	1.2	7.15	–	543	44,350,000	–	90
72 hours	–	–	111.5	119.8	–	1.2	7.2	–	–	15,400,000	–	90
84 hours	–	–	114.4	123.4	–	1.15	7.2	–	–	6,150,000	–	90
96 hours	–	483	120.0	94.6	48.6	1.15	–	–	–	875,000	90% Gram – : 5% shadows : 5% spores	80
7 days	635.0	476	129.0	93.8	62.0	–	–	–	–	665,000	–	–
Fermented veal-infusion pepton + 2 per cent glucose												
0 hours	573.3	353	125.8	53.4	19.2	0.5	7.13	2.0	0	1,900	100% Gram –	0
12 hours	–	–	121.0	–	–	0.6	7.16	2.0	0	176,500	96% Gram + : 4% Gram –	0
18 hours	–	–	144.0	44.6	–	1.2	6.74	1.87	15	174,500,000	–	0

24 hours	–	–	146.0	42.1	–	1.4	6.61	1.80	118	97,500,000	–	25
30 hours	–	–	128.6	36.4	32.4	1.5	6.41	1.39	1025	2,315,000	40% Gram + : 60% Gram –	5
36 hours	–	–	164.7	44.8	–	1.1	6.30	1.27	1150	8,050,000	44% Gram + : 56% Gram –	25
42 hours	–	–	141.8	49.6	–	1.6	–	1.05	1380	–	–	35
48 hours	–	378	164.7	39.1	41.8	–	6.15	0.93	1740	3,847,500	–	25
54 hours	–	–	–	39.1	–	–	–	0.61	2121	–	–	–
60 hours	–	–	154.5	44.8	–	1.8	5.95	0.38	2620	2,900,000	–	50
72 hours	–	–	154.5	47.0	–	–	–	0.26	3098	10,400,000	–	0
84 hours	–	–	154.0	49.0	–	1.5	5.95	0.26	3660	146,250	95% Gram + : 5% shadows	0
96 hours	–	434	154.0	–	54.0	–	5.95	–	4140	2,775,000	60% Gram + : 40% Gram –	0
7 days	593.0	434	189.0	46.1	–	–	–	0.16	–	290,250	–	–

Fermented veal-infusion-pepton + 2 per cent glucose + 1 per cent phosphate

0 hours	583.0	364	117.5	49.0	21.0	0.75	7.15	1.87	0
12 hours	–	–	116.4	49.0	–	–	7.05	1.87	60
18 hours	–	–	124.0	58.7	–	1.55	7.00	1.8	131
24 hours	–	–	155.0	41.0	–	1.7	6.71	1.26	311
27 hours	–	–	–	–	–	–	–	–	1620
30 hours	–	–	150.0	–	36.0	1.85	6.74	0.92	2210
36 hours	–	–	145.0	–	–	2.25	6.46	–	2580
42 hours	–	–	145.0	39.3	–	2.35	6.55	0.49	2900
48 hours	–	378	164.0	–	39.0	2.25	6.56	0.39	3410
54 hours	–	–	164.0	–	–	1.8	6.35	0.32	3710
60 hours	–	–	164.7	–	–	–	6.39	0.14	4165
72 hours	–	–	154.0	–	–	1.5	6.29	–	4376
84 hours	–	–	161.0	36.9	–	2.1	6.29	0.07	–
96 hours	–	402	185.0	–	44.0	2.3	–	0.00	–
7 days	605.0	462	199.5	78.3	49.0	–	–	–	–
–	–	–	–	80.6	–	–	–	–	–

has been noted, originated from "bob" veal. This striking difference in nitrogen content of the two media serves to emphasize the desirability of a chemical analysis of culture media in bacteriologic work. The difference in biologic value of the two media after fermentation with *B. welchii* is reflected in the plate counts, which show a ten-fold greater number of viable organisms in the medium richer in nitrogen as may be noted from tables 14 to 15.

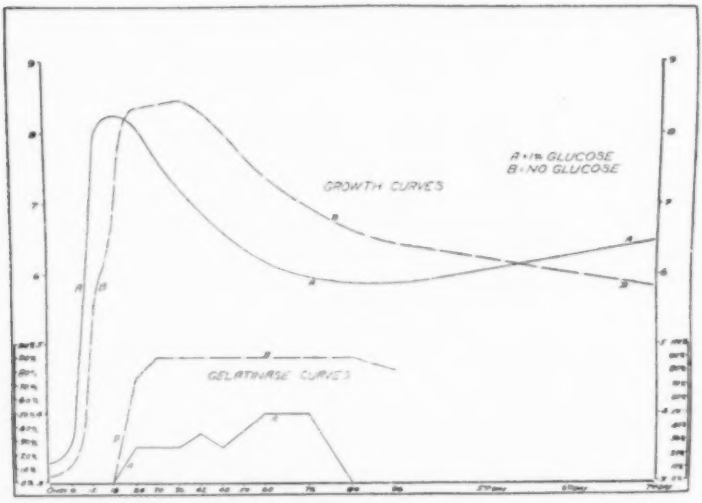


CHART 10. THE EFFECT OF GLUCOSE ON GROWTH AND GELATINASE

The 543 cc. of gas formed in the sugar-free medium could only have come from the metabolism of the protein molecules. Hall (1921) reported only "traces" of gas formed by *B. botulinus* in sugar-free media. The discrepancy in results is probably not unrelated to the six-day fermentation of *B. welchii* to which the medium he used had been subjected.

The most conspicuous effect produced by the addition of 2 per cent glucose was on the death rate of the organisms. The medium without sugar not only supported a greater number of organisms at the height of growth, but the death rate was very much lower during the first three days of incubation. A graphic

representation of this loss of biologic value by the addition of the sugar is shown in chart 10. A comparison of the reaction of the two media shows that the sugar-free substratum remained at approximately the optimum reaction for *B. botulinus*, while the acid products of sugar fermentation caused an increase in acidity from pH 7.13 to 5.95, and this in a medium which had the not insignificant buffer index of 5.5. On the forty-eighth hour of incubation the sugar had spared the protein considerably, as shown by non-protein nitrogen determinations, but this sparing effect was less evident on the seventh day of incubation. A moderate sparing effect is also suggested by a greater accumulation of amino acids in the sugar-containing, and a greater accumulation of ammonia in the sugar-free media. The volume of gas, as was expected, was more than 7 times as large in the former case.

The addition of phosphate increased the buffer index from 5.5 to 10, and in consequence the reaction was depressed to pH 6.29 instead of 5.95. The slightly greater quantity of gas was formed at a more rapid rate. There was no opportunity for plate counts, but a considerable increase in non-protein and ammonia nitrogen after the exhaustion of the same amount of sugar as in the phosphate-free medium, suggests the preservation of more viable organisms. There was no lag or inhibition in gas production, such as Wolf (1920) found. In fact, the results show a somewhat more rapid evolution of gas.

The very similar amounts of volatile acids in the three media on the seventh day of incubation is evidence of their origin from sources other than carbohydrate.

Effect of glucose on gelatinase production. The results recorded in table 15 and represented graphically in chart 10, are very clear cut with regard to the mooted question of gelatinase production by *B. botulinus*. Van Ermengem reported gelatin liquefaction by cultures of the Ellezelle strain of this organism. Kendall, Day and Walker (1922) do not find such liquefaction, a conclusion not confirmed by Hall (1922), Kahn and the British Medical Research Committee (1919). The recently isolated Types A and B strains of *B. botulinus* making up the large collection in this laboratory consistently liquefy gelatin when grown in gelatin-

containing media. The technic employed in the experiments herein reported was essentially that used by Jordan (1906) and later by Kendall and Walker (1915). The cultures were centrifuged for forty-five minutes at high speed and 1 cc. of the supernatant fluid was added to 9 cc. amounts of solidified carbol-gelatin. The seed tubes were incubated at 37°C. for forty-eight hours, and after a subsequent twenty-four hours of refrigeration at 8°C. readings were made on a basis of percentage liquefaction.

The appearance of a gelatin liquefying enzyme could not be demonstrated in either culture until after the eighteenth hour, although considerable growth had occurred in both by that time. The gelatinolytic potency of the sugar-containing culture was from a third to a half less than that of the sugar-free one and by the seventy-second hour of incubation the liquefying power of the former had disappeared, while that of the latter was still at its height. At the end of ninety-six hours there was demonstrable only a slight loss of potency in the sugar-free culture. There is no evidence of the relation of reaction of the sugar-containing medium to the loss of gelatinolytic potency, which is in harmony with the conclusion of Jordan (1921).

The greater gelatinase production by *B. botulinus* in the sugar-free medium, is in harmony with the results of Fermi, Schmailowitsch, Matzuschita, Kendall and Walker, and Jones (1916, 1920), who found proteolytic enzymes in greatest quantity in protein media free from sugar. The results do not harmonize however with those of the last two workers in that they found the presence of utilizable sugar entirely prevented the appearance of proteolytic enzymes. Jordan did not find this to be true, however, and Blanc has published curves showing that the presence of glucose accelerated the appearance of a gelatinolytic enzyme in *B. histolyticus* cultures, and did not prevent its appearance in cultures of *B. sporogenes*. While the figures listed in table 15 reveal a greater gelatinase production by the sugar-free culture of *B. botulinus*, it should be kept in mind that plate counts also revealed a much lower level of growth in that medium, and it may well be that the gelatinolytic potency of the two

cultures is correlated closely with the amounts of growth the media supported.

The loss of biologic value after the addition of glucose is in striking contrast to the results reported for such an addition to equal parts of veal-infusion and peptic-digest (Dozier, Wagner and Meyer). The increase in the biologic value of the latter was clearly indicated by the larger number of organisms at the height of growth and also by the maintenance of a higher level for a longer time. This medium was 30 per cent richer in amino nitrogen while the total nitrogen was only about 10 per cent higher, making it probable that the form of the nitrogen had more influence in causing these differences in growth than did the total nitrogen content. The unfermented veal of the earlier experiment undoubtedly contributed a small amount of sugar, and possibly other substances, which stimulated growth. A comparison of the initial buffer indices of the media used in the two experiments is of interest. The veal-infusion peptic-digest carried an inherent buffer index of 11.7, in contrast to the 5.5, of the veal-infusion 1 per cent pepton, and undoubtedly added a quota to the higher biologic value of the former.

Experimental series 13

Visual observations of milk cultures of bacteria are of some interest in that the pictures presented may give a clue to the chemical changes occurring. The macroscopic appearance of milk cultures of *B. botulinus* has been noted and described by numerous workers. Such observations, however, have, in but few cases, been accompanied by chemical studies confirming the hypotheses advanced as to the probable course of metabolism. The detailed analyses reported by Kendall, Day and Walker (1922) are a notable exception; their results offer evidence of a relatively slight metabolic activity by *B. botulinus* on this medium.

Van Ermengem's Ellezelle strain of *B. botulinus* failed in its discoverer's hands to exhibit any signs of metabolic activity in milk. V. Hibler (p. 99), on the contrary, found precipitation of casein with subsequent peptonization in cultures of that strain

as well as in those of two others. The British Medical Committee (1919) record the lack of any action on milk. Kendall, Day and Walker (1922) failed to note coagulation of the casein in any of their cultures. Thom, Edmondson and Giltner report a "fine coagulum, with peptonization beginning about the fifth day of incubation." It should be noted, too, that they report a slight reddening of litmus milk, with subsequent decolorization. Shippen found acidification and peptonization of milk. Nevin found milk coagulated in three days, as did Armstrong, Story and Scott who report decolorization of litmus milk cultures in two to three days, followed later by peptonization. Kahn found a firm coagulum which was subsequently almost completely digested, but he failed to observe gas in his cultures, while Hall (1922) reports slow coagulation followed by digestion and gas production. These conflicting statements are reflected in recent text-books.

Eighteen hundred cubic centimeters of fresh certified milk, with cream removed, were placed in Erlenmeyer flasks and on three successive days heated quickly in a boiling water bath and held at 95° to 100°C. for thirty minutes in an Arnold sterilizer. This procedure was adopted in order to preserve so far as possible the original chemical composition of the milk constituents. Heinemann (p. 131) advises that milk sugar begins to decompose at 120°C., liberating acids. Clark (1915) autoclaved milk for fifteen minutes and found a distinct increase in acidity, while prolonging the heating to thirty minutes caused reactions as acid as pH 6.13. The reaction of the milk, sterilized as just described was pH 6.6, which is in harmony with the reactions found in fresh milk by various investigators. Van Slyke and Baker tested 300 samples of freshly drawn milk and found over 80 per cent of this number had a H-ion concentration between pH 6.5 and 6.8, inclusive. The color of the milk was not altered, which is additional proof of freedom from caramelization. The effect of heat of sterilization upon the integrity of the nitrogenous constituents of milk is largely problematical. The amino acid content of sterilized milk has been found in this laboratory to be considerably higher than the 2.6 to 7.3 mgm. per 100 cc. reported by Denis and Minot for unheated market milk. Their

figures approximate those which Wolf and Harris (1917) ascribe to milk sterilized in the autoclave. On the other hand Kendall, Day and Walker (1922), Bushnell and Spitzer and Weeter report higher figures, which more nearly approach those found in this study.

The sterilized milk was seeded with spores, inoculations of 5 cc. from heated meat cultures of *B. botulinus* strains 38 and 97, Type A. Subsequent observations had shown that the growth processes of *B. botulinus* on milk were slow in comparison with those on meat media, and in order to detect, if possible, the initiation of chemical changes, the physical appearance of the media was carefully watched particularly during the early hours of incubation. At the end of thirty-six hours the milk appeared unaltered; after sixty hours of incubation clotting and considerable syneresis had occurred; the whey of strain 38 appeared greenish-yellow and that of strain 97 golden-yellow. At the end of one hundred eight hours the colorations were much the same; and on the seventh day the curds were markedly digested. At the end of thirty-six hours the viable organisms in the culture of strain 38 had increased from 1780 to 33,250,000 per cubic centimeter. This marked proliferation was not predictable by odor or general appearance. Practically the same behavior was noted for strain 97.

While the chemical changes[5] during the first thirty-six hours do not seem significant, both cultures show a loss in amino acid nitrogen, accompanied by slight increases in the ammonia nitrogen, which is evidence of protein synthesis. The results as to volatile acid, titratable acid and gas determination are in harmony, while the reaction of strain 38 had changed from pH 6.6 to 6.2. Strain 97 exhibited a slight increase in acidity.

Eighty-fourth hour determinations included estimation of nonprotein nitrogen, which show much greater proteolytic activity

[5] The methods of chemical analysis differed from those previously described in that determinations for ammonia, amino acid, volatile acid and sugar were all made on protein-free filtrates. Caustic NaOH was used for the subsequent ammonia determinations. The greater efficiency of this reagent was discussed by Wagner, Doxier and Meyer.

in both cultures, than would be predictable by either amino acid or ammonia nitrogen increases, or even the sums of the two. By the end of seven days over 70 per cent of the nitrogen of the milk had been reduced to non-protein form. The ammonia and amino acid nitrogen increased consistently until at the end of twelve days incubation, 57 per cent and 63 per cent, respectively, of the total nitrogen of cultures 38 and 97 being converted into

TABLE 16

Growth of B. botulinus in sterile milk

TIME	TOTAL N	NON-PROTEIN N	AMINO N	AMMINO N	VOLATILE ACID N/10	TITRATABLE ACID N/1	pH	SUGAR	GAS PER LITER	ORGANISMS PER CUBIC CENTIMETER
B. botulinus strain 38										
	mgm.	*mgm.*	*mgm.*	*mgm.*	*cc.*	*cc.*	"C"	*per cent*	*cc.*	
0 hours	567	42.0	31.2	8.8	3.0	1.4	6.6	4.69	0	1,780
36 hours	—	—	21.3	15.6	6.2	2.2	6.2	4.55	123	33,250,000
60 hours	—	—	25.0	23.8	8.2	3.4	6.0	4.55	249	14,200,000
84 hours	—	252.0	54.1	42.0	13.6	4.0	6.0	4.55	682	17,300,000
96 hours	—	—	—	—	—	—	—	—	874	—
108 hours	—	—	84.4	82.8	21.4	4.6	6.0	4.66	—	65,000,000
132 hours	—	—	—	—	—	—	—	—	1120	—
7 days	—	406.0	122.6	129.9	39.6	5.0	6.2	4.80	1120	42,500,000
12 days	—	—	157.5	162.5	50.0	5.6	6.62 E	—	—	—
B. botulinus, strain 97										
0 hours	567	42.0	31.2	8.8	3.0	1.4	6.6	4.69	0	—
12 hours	—	—	—	—	—	—	—	—	—	132,000
36 hours	—	—	12.8	14.3	3.2	1.6	6.6	4.69	68	30,250,000
60 hours	—	—	23.0	15.6	10.4	3.7	6.0	4.57	162	39,000,000
84 hours	—	273.0	58.0	75.0	19.8	4.2	6.0	4.57	392	142,000,000
96 hours	—	—	—	—	—	—	—	—	543	—
108 hours	—	—	80.2	111.5	27.4	4.5	6.2	4.66	616	89,000,000
132 hours	—	—	—	—	—	—	—	—	940	—
7 days	—	406.0	108.5	150.5	40.8	4.8	6.4	4.80	1020	46,500,000
12 days	—	—	186.0	172.0	48.0	5.2	7.0 E	—	—	—

such products. The amounts of volatile and titratable acids show a steady increase, as do those for gas production. The reaction curves for the two organisms are quite similar, showing first an increase in acidity from pH 6.6 to 6.0, and then a gradual decrease until neutrality was reached by strain 97 on the twelfth day, while strain 38 had reached a pH of 6.62 at the same time.

Plate counts show a slow growth until on the one hundred eighth hour strain 38 had a maximum of 65,000,000, and on the eighty-fourth hour strain 97, 142,000,000 viable organisms per cubic centimeter. The counts on the thirty-sixth hour in each case indicate a greater proliferation than might have been anticipated from the chemical determinations. The death rate of the organisms was comparatively slow. In this connection it may be mentioned that the toxicity of the milk culture of strain 38 has shown a comparatively slow accumulation of poison.

Sugar consumption. The production of acid by *B. botulinus* during the first stages of growth on milk has been noted consistently in this laboratory, and the figures in table 16 reveal a drop in pH in both flasks from pH 6.6 to 6.0. The origin of the acid presents an interesting problem. Conflicting reports on the ability of *B. botulinus* to utilize lactose will be summarized by Meyer and Oman. They failed to secure lactose fermentation in test cultures.

Elser and Huntoon found differences in the lactose utilization by meningococci dependent on the method of sterilization, and more recently Wolf (1921) has suggested that when lactose is sterilized in combination with bouillon it is quite possible that a molecular rearrangement may result enabling certain organisms to utilize the milk sugar molecule.

Theobald Smith (1898) from bacteriological evidence, attributed the initial acidity of the Colon-typhoid-dysentery group of bacteria to the fermentation of a glucose-like substance present in milk to the amount of about 0.1 per cent. Jones, in 1914, concluded, "Bacteriological and chemical evidence . . . . indicates that milk normally contains a substance which reacts like dextrose." These conclusions have been accepted by Hall (1921), by Kendall (1923), and by Hiss, Zinsser and Russel (p. 646). However, Raudnitz, in 1909, stated his conviction that lactose is the only carbohydrate in milk.

Hall (1921) has suggested the probability of slow hydrolysis of lactose in the presence of ammonia produced by putrefactive anaerobes until there is " . . . . the almost complete destruction of the high lactose content of the milk," but this

is a hypothesis for which we have no supporting evidence so far as it concerns either *B. botulinus* or *B. tetani* cultures. Mention may be made of reactions as acid as pH 6.0, which have been found in 12 day milk cultures of *B. tetani* by one of the authors (E. W.). This is as great an increase in free H-ions by a non-glucose fermenting organism as has been found for the glucose-fermenting *B. botulinus*. Successive sugar determinations, as listed in table 16, show a constant level of reducing substances in the *B. botulinus* cultures. The slight variations may well lie within the limits of experimental error. The possibility that the lactose molecules were slowly hydrolyzing to glucose and galactose, and that the former were immediately destroyed by the botulinus bacilli must be considered. The galactose molecules would exert the same reducing power as the intact lactose, and the net result would be the same as that given in table 16. This hypothesis is not supported, however, by the comparatively slow evolution of gas. In an effort to throw light upon this very complex question the following experiment was undertaken.

Experimental series 14

Milk of the same grade as that previously used was sterilized in an autoclave at 115 pounds pressure for forty-five minutes. The color suggested slight caramelization, which the pH of 6.4 confirms. To one flask enough sterile 50 per cent glucose solution was added to make a concentration of 1.25 per cent. An equal amount of physiological saline was added to the other flask. Strain 38 was inoculated into the flasks, and both were incubated and sampled under identical conditions.

Initial analyses revealed a lactose content of 4.14 per cent. Unsuccessful attempts were made to hydrolyze all of the lactose to hexoses as soon as samples were removed. Had these been successful subsequent determination of the reducing power would have given adequate information to answer the question involved; namely, is any of the milk sugar utilized by *B. botulinus?* The experiment, however, furnished considerable circumstantial evidence that no part of the lactose molecule is metabolized during the growth of *B. botulinus*. The flask con-

taining the added glucose yielded nearly nine times as much gas as the sugar-free culture on the one hundred fourteenth hour of incubation. This culture became progressively more acid until a pH of 5.50 was reached in contrast to the pH of 6.18 reached in the plain milk. The ammonia curves of the two media furnish corroborative data: 54.4 mgm. of ammonia nitrogen per 100 cc.

TABLE 17

B. botulinus on milk with and without added glucose

TIME	AMINO N	AMMONIA N	VOLATILE ACID N/10	pH	LACTOSE	GLUCOSE	GAS PER LITER
			B. botulinus, strain 38				
	mgm.	*mgm.*	*cc.*	"E"	*per cent*	*per cent*	*cc.*
0 hours	32.0	3.4	4.0	6.4	4.14	1.25	0
18 hours	38.0	—	—	6.34	—	—	138
42 hours	41.0	15.3	12.0	—	—	—	171
54 hours	—	—	—	—	—	—	494
78 hours	70.5	—	—	—	—	—	1820
90 hours	—	—	19.8	5.75	—	—	2023
114 hours	—	—	—	—	—	—	2510
162 hours	135.0	45.9	29.2	5.58	—	—	2750
10 days	171.0	54.4	41.0	5.5	—	—	—
			B. botulinus, strain 38				
0 hours	30.0	3.4	4.0	6.4	4.14		0
18 hours	37.5	—	—	6.38	—		10
42 hours	34.6	14.4	6.4	—	—		34
54 hours	—	—	—	—	—		—
78 hours	—	—	—	—	—		44
90 hours	32.8	15.5	7.2	6.25	—		117
114 hours	—	—	—	—	—		282
162 hours	120.0	95.2	28.8	6.18	—		466*
10 days	178.0	110.5	40.0	6.18	—		—

* Gas lost in the measuring cylinder.

of medium had accumulated in the culture containing the added glucose compared with 110.5 mgm. per 100 cc. in the plain milk culture, showing the protein-sparing action furnished by utilizable carbohydrates.

Enzymes in milk cultures. The conflicting opinions in the literature regarding the coagulating and casein digesting abilities of *B. botulinus* have been mentioned. In this laboratory *B.*

botulinus has consistently coagulated the casein of milk, and slow digestion has followed. Further experimental proof of coagulating power was secured by tests for a coagulating enzyme. One cubic centimeter amounts of sterile filtrates from the milk cultures of strains 38 and 97 were introduced into 9 cc. of sterile milk and incubated at 37°C. Readings after twenty-four hours revealed clotting in every case.

Casein digesting powers of *B. botulinus* were tested by the use of milk plates, after the method of Eijkman. About 10 per cent of sterile milk was added to glucose-liver agar, inoculated with *B. botulinus*, poured into plates and incubated at 37°C. for forty-eight hours in the anaerobic jars used for blood agar plates. Clear areas surrounding each colony attested to the presence of a casein-splitting enzyme. Streak cultures on milk plates were always characterized by a digested area on either side of the line of growth.

A third test for enzyme action was performed by filtering a sample of the milk culture of strain 38 and adding 1 cc. of the sterile filtrate to 2 per cent Difco and Witte's pepton solution, which by the usual determinations were shown to have 64 and 30 mgm. respectively of amino nitrogen per 100 cc. These were incubated at 37°C. for thirty-six hours and the analyses repeated. The amino acid nitrogen contents increased to 85.2 and 72.4 mgm. per 100 cc., respectively, revealing a very definite hydrolyzing capacity on the part of such sterile filtrates.

It should be recalled that the presence of a gelatinase in *B. botulinus* cultures has previously been demonstrated.

Experimental series 15

Growth of B. botulinus and B. tetani on casein digest. Casein digest was prepared by the method of Cole and Onslow from commercial casein. Total nitrogen determinations on the digested mass after filtration were made and a solution having a nitrogen concentration comparable to that of the fresh milk used in the previous experiments was prepared by the aid of such data. A picture of the approximate stage of digestion of the protein may be obtained by reference to the initial figures for total, non-pro-

TABLE 18

Growth of B. botulinus and B. tetani in casein digest

TIME	TOTAL N	NON-PROTEIN N	AMINO N	AMMONIA N	VOLATILE ACID N/10	TITRATABLE ACID N/1	NON-VOLATILE ACID N/10	pH	GAS PER LITER	ORGANISMS PER CUBIC CENTIMETER
						B. botulinus, strain 38				
	mgm.	*mgm.*	*mgm.*	*mgm.*	*cc.*	*cc.*	*cc.*	"E"	*cc.*	
0 hours	591	539	220	36	6.0	2.1	4.8	7.28	0	87,000
12 hours	—	—	220	36	—	2.2	—	7.28	34	247,000
24 hours	—	—	231	58	9.0	3.3	—	7.25	80	9,100,000
36 hours	—	—	212	132	43.6	3.4	—	7.41	137	81,500,000
48 hours	591	574	199	157	58.8	1.8	—	7.3†	311	187,000,000
60 hours	—	—	193	172	66.0	1.4	19.6	7.2†	415	74,000,000
72 hours	—	—	196	174	68.0	3.3	—	7.2†	561	40,000,000
96 hours	607	598	196	176	68.0	1.6	—	7.3†	—	94,000,000
10 days	—	—	205	182	80.0	1.3	23.8	7.4†	—	—
30 days	—	—	180	196	72.0	1.3	33.8	6.8†	—	—
						B. botulinus, strain 97				
0 hours	591	539	220	36	6.0	2.1	4.8	7.25	0	125,000
12 hours	—	—	232	53	9.2	2.4	—	7.2	94	28,900,000
24 hours	—	—	218	127	45.4	3.1	—	7.53*	259	322,500,000
36 hours	—	—	190	160	68.6	3.8	—	7.4	467	219,250,000
48 hours	591	574	168	195	91.6	2.0	—	7.2	630	144,500,000
60 hours	—	—	181	205	94.0	1.4	22.8	7.04	733	21,700,000
72 hours	—	—	182	208	94.0	3.3	—	6.98	—	23,725,000
96 hours	585	574	193	212	—	2.1	—	6.8†	—	8,475,000
10 days	—	—	183	233	110.0	1.4	28.4	7.2†	—	—
30 days	—	—	158	260	108.0	1.4	—	6.6†	—	—
						B. tetani V. T. S.				
0 hours	588	511	220	36	6.0	2.1	4.8	7.25		14,850
12 hours	—	—	214	36	—	2.2	—	7.25		20,500
24 hours	—	—	221	36	—	—	—	—		—
36 hours	—	—	221	—	—	—	—	—		156,000
68 hours	—	—	—	—	—	—	—	—	—	—
40 hours	—	—	—	—	—	—	—	—		23,700,000
72 hours	—	—	216	48	10.8	2.8	—	7.05		—
96 hours	581	546	199	104	43.0	2.3	—	6.9†		304,500,000
7 days	—	—	—	—	—	—	14.8	—		—
10 days	—	—	183	140	69.2	1.2	—	—		—
20 days	—	—	183	158	69.0	—	—	7.0†		—

* Determinations made after holding in ice box 36 hours.

† Determinations made colorimetrically.

tein, amino acid and ammonia nitrogens listed in table 18. Of the 591 mgm. of nitrogen per 100 cc. of the finished medium 539 mgm., or 91 per cent, were resident in non-protein molecules, and half of this was found in the amino acid and amonia fractions. As would be expected from these tests, the medium was not found to be "biuret free."

Plate counts furnish evidence of the greater biologic value of this medium than had been shown for milk, a property which in large part may reasonably be ascribed to its easily available nitrogen. The possibility that the lack of such nutrients may account for what some workers believe to be the germicidal action of fresh milk has occurred to the writers.

Strain 97 grew more vigorously than strain 38. The data contained in table 16 support this contention. The smaller amounts of gas recorded for the casein digest medium are more apparent than real. Growth processes were initiated much earlier in the former case and as a result sampling was much heavier during the first days, which resulted in an increased gas space. This lessened the length of the interim during which accurate measurements of gas could be made.

Buffer index determinations were made just after inoculation and at the end of ninety-six hours. During this time the buffer capacity had increased from 5.4 to 8.1 and 9.1, respectively, for strains 38 and 97. The pH of the former culture had remained quite stationary, while that of the latter had decreased from 7.25 to 6.8 in spite of the greater buffer capacity which had developed. This is additional evidence of the greater vigor of strain 97.

Attention may be called to the definitely larger amounts of titratable acid which developed in the milk than in the casein digest cultures; and to their steady increase in the former in contrast to the rise and fall of such products in the latter. No attempt is made at this time to explain these findings.

Comparison of the metabolism of B. botulinus with B. tetani. The growth curves of the two cultures of *B. botulinus* and of *B. tetani* are shown graphically in chart 11. There was a very much longer latent period in the latter, but as high a level of growth was finally attained as is shown for either of the strains of *B. botulinus*.

The buffer index changed from 5.4 to 8.3, which is more of a change than strain 38 produced and less than that which occurred in the culture of strain 97. This, of course, suggests that the hydrolytic power of *B. tetani* is less than that of strain 97 and greater than that of strain 38. The height of amino acid accumulation was not very different from that shown by the *B. botulinus* cultures. The ammonia nitrogen accumulation was

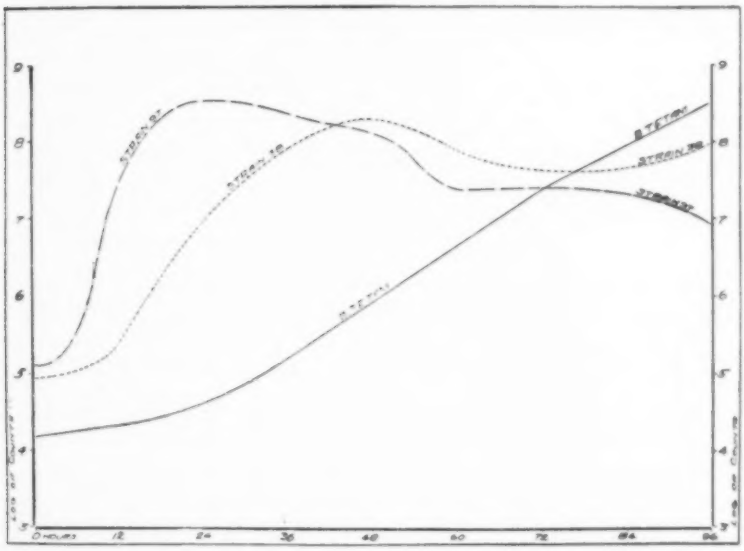


CHART 11. GROWTH OF B. BOTULINUS AND B. TETANI IN CASEIN DIGEST MEDIA

not so great, nor was there as great an increase in volatile acid as that produced by strain 97, but it reached about the same level as that of strain 38. The pH of the *tetanus* culture changed from 7.25 to 6.9 and even on the twentieth day of incubation had reverted to 7.0 only.

As a whole the results are not out of harmony with the previous conclusion that *B. tetani* is as proteolytic as *B. botulinus* (Wagner, Dozier and Meyer), although the nature of the medium is not such as to allow a sweeping conclusion.

The increase in H-ion concentration again presents the phenom-

TABLE 19

Volatile acid produced in casein digest

	10 DAY CULTURE					
	Strain 38, *B. botulinus*		Strain 97, *B. botulinus*		Strain V.T.S., *B. tetani*	
	First fraction	Second fraction	First fraction	Second fraction	First fraction	Second fraction
N/10 Barium hydroxide to neutralize volatile acid cc	17.9	7.8	25.6	14.8	11.3	9.9
Volatile acids in fractions of distillate per cent — 10 cc	27.4	12.8	21.9	11.5	14.2	10.1
20 cc	45.7	24.4	38.7	22.3	26.7	20.2
30 cc	60.9	33.3	51.5	33.1	38.0	29.3
40 cc	70.8	42.4	63.0	42.0	48.6	39.4
50 cc	78.8	57.4	73.5	50.6	58.5	48.5
60 cc	84.4	60.3	79.0	59.5	66.5	58.6
70 cc	88.8	73.0	85.2	68.2	76.0	67.6
80 cc	92.6	78.2	90.6	77.0	83.0	77.8
90 cc	96.1	90.0	95.3	86.5	91.2	88.0
100 cc	100.0	100.0	100.0	100.0	100.0	100.0
Ratio of acids present from titration sequence	4 valeric, 1 acetic	1 butyric, 2 acetic	2 valeric, 1 acetic	1 butyric, 2 acetic	1 butyric, 1 acetic	1 butyric, 3 acetic
Volatile acids as barium salts	0.2625	0.1107	0.3889	0.2035	0.1631	0.1297
$BaSO_4$ equivalent found	0.1956	0.0890	0.2882	0.1672	0.1238	0.1084
Barium salt calculated from ratio of acid found by titration	0.270	0.1108	0.385	0.1985	0.1480	0.1253
Difference between volatile acid barium salts found and calculated	0.0075	0.001	0.0039	0.0050	0.015	0.0044

enon which was discussed in connection with a decrease in pH and the lack of any other evidence of sugar fermentation by *B. botulinus* in milk and casein digest cultures. In this case, however, not only may the medium be assumed to be sugar-free, but there is the added assurance that no fermentation has occurred which the well-known lack of any carbohydrophilic tendency on the part of *B. tetani* confirms.

Nature of the acids formed by B. botulinus and B. tetani in milk and in casein digest. The volatile acids in seven- and ten-day milk and casein digest cultures, respectively, were extracted and identified. Details for the milk cultures are omitted. Valeric, butyric and acetic acids in the proportion of 4:1:5 were identified in the milk culture of strain 38, and in the proportion of 5:3:4 for strain 97. The data for the casein digest cultures are listed in table 4. They indicate that a mixture of valeric, butyric and acetic acids was present in the approximate proportion of 2:1:2 and 2:1:3 in the cultures of *B. botulinus* strains 38 and 97, respectively, while in the *B. tetani* culture butyric and acetic acids were present in the ratio of 3:5. These results, as a whole, agree fully with those reported in previous experimental series, and emphasize again the difference in the nature of the volatile acids formed by *B. botulinus* and *B. tetani*, or in other words, the specificity of the metabolism of the two species of organisms. The comparatively large amounts of such acids in the casein digest medium, undoubtedly sugar-free, are additional proof that the volatile acids have their origin in protein decomposition products. It seems reasonable to the writers to credit at least a large part of the increase in H-ion concentration recorded for the milk and casein digest cultures to the volatile acid accumulations. There is also a definite increase, as may be noted from table 18, in fixed acids, which curiously enough, were identified in both cultures as lactic acid, and one is reminded of the usual graphic representations of the inter-relations between carbohydrate and protein metabolism which show the path to be through lactic acid.

DISCUSSION

The investigations detailed in the preceding paragraphs have revealed a number of facts, which deserve some discussion. In order to make the study as comprehensive as possible the chemical activities of *B. botulinus* have been studied in a number of media. These data permit more readily of general conclusions than do those, which were secured by Kendall, Schübel and others on a few selected and probably unsuitable media.

B. botulinus is not particularly exacting in its food needs. Preliminary experiments have indicated that the organism will grow under anaerobic conditions in liver, brain, serum, bean, olive, corn, meat-mash, pepton, milk and digest media. In bean and olive infusions however the growth and the chemical changes were not sufficiently striking to make them satisfactory substrata for a biochemical study.

B. botulinus Type A and B produce gas in any medium which supports good growth just as *B. sporogenes* does (Wolf and Harris). The accumulation of gas is greatly increased by the addition of glucose to the substratum. In poorly buffered media this fermentation produces an acid reaction, which in some cultures may inhibit the growth and even the toxin production. However the carbohydrate metabolism is not concerned in the production of the volatile acids, which are mainly composed of butyric, valeric and acetic acids. The organism is strongly proteolytic in absence of oxygen.

The toxin production is apparently intimately connected with the enzymatic activity which develops in the culture. The nature of the enzymes is still under investigation. Studies conducted by Mrs. Forster have shown that secondary and tertiary proteases (Bradley and Dernby) are probably responsible for the nitrogen breakdown in the cultures. Furthermore it is established that the ferments are only mobilized when needed. For example no gelatinase was demonstrable in a spinach infusion culture. No separation of the products resulting from enzyme action and bacterial growth can be made; however, ammonia, volatile acids and gaseous products have not been detected as a result of enzyme action.

These observations immediately suggest the question, what are the underlying chemical or enzymatic reactions responsible for the production of the botulinus toxin and what is the chemical composition of the poison in the light of our present knowledge. One enters here into the field of hypothesis. It is needless to emphasize that the views which are presented serve merely as a guide for studies already in progress.

The recent work of Landsteiner and Lampl leaves little doubt that the antigenic properties of toxins are attributable to specific, relatively simple protein compounds. It is reasonable to assume that the botulinus toxin is either an intermediary protein decomposition product or it is intimately connected with such a substance. According to Bronfenbrenner it is a mono-valent, negatively charged ion. Schübel reports on the diffusion of the toxin through parchment paper and collodion membranes. In one experiment the dialysate failed to give a biuret reaction. These observations have not as yet been confirmed; in fact Coleman concludes from some experiments with collodion sacs in the peritoneal cavity of guinea-pigs that the toxin is non-diffusible. Observations reported by Schübel and confirmed in this laboratory lend little support to the hypothesis that the botulinus toxin is either a lipoid or specific fat complex in the sense of Warden, Connell and Holly.

Regarding the mechanism of the toxin production two possibilities must be considered. The toxin is either of endogenous or of exogenous origin. In discussing the possibility of an endogenous source a number of facts and interpretations merit attention:

a. The toxin may be a normal excretory product comparable with the urea of vertebrates. The fact that growth and toxin formation rarely run parallel does not support this hypothesis. Excellent growth is not always followed by a potent toxin production.

b. The toxin may be an abnormal product of bacterial metabolism, which is only formed provided the organisms have available certain amino acids or growth accessory substances. Numerous observations lend some support to this contention. As far as

published and unpublished records permit of conclusions it is evident that *B. botulinus* cannot multiply in synthetic media. However, very dilute solutions of lower protein degradation products encourage the production of toxin. In fact a biuret free aminoids solution yields a stronger toxin than a pepton medium which is rich in albumoses. Some experiments reported in this paper encourage the belief that the toxin is only formed in the presence of accessory factors. For example Berna pepton, incorporated in a veal-infusion furnishes a very strong toxin, while the pepton solution alone stimulates a moderate growth and little or no toxin. On the other hand it must always be remembered that toxin production can take place in any vegetable extract, provided the anaerobe can grow.

c. The toxin may be set free through the autolysis of the bacteria. C. C. Dozier in this laboratory has enumerated a number of facts which indicate that the botulinus bacilli may serve as the matrix of the poison. Her observations have been verified but additional experiments are necessary to confirm her interpretations.

The evidence substantiating the endogenous origin of the botulinus toxin is in many respects very suggestive, but the literature furnishes also a number of examples which favor the exogenous origin of the poison. Kendall considers the toxins of *B. diphtheriae* or *B. tetani* as by-products formed during the forced utilization of protein for energy. Dzierzgowski and Rekowski concluded that the toxin of the diphtheria bacillus was formed from proteoses or higher polypeptids of the medium.

More recently two hypotheses, the "protoxin theory" of Morgenroth and of Walbum and the "enzyme theory" of Dernby and Walbum dealing with the toxin of *B. diphtheriae* have attracted some attention. Walbum (1909) demonstrated that diphtheria toxin and staphylolysin were not formed within the bacteria themselves, but that there was a previous stage—a sort of atoxic protoxin. These studies have been carried further by Dernby and Walbum. An extract of a young diphtheria culture does not possess a very high toxicity. In one experiment it corresponded to 250 toxic units per cubic centimeter. If this

feebly toxic extract was mixed with a solution of Witte's pepton and the mixture placed in the incubator, the toxicity increased in the course of twenty hours to more than 600 toxic units per cubic centimeter and then decreased again. A still stronger reaction was produced if, instead of the Witte's pepton solution alone, the bacterial extract was mixed with ordinary pepton-bouillon, when the toxicity was seen to increase to 1000 units in the course of ten hours, and to fall rapidly afterwards. Similar experiments have not as yet been carried out with *B. botulinus*, but independent observations made by several workers indicate that the toxin of *B. botulinus* may behave similarly to that of *B. diphtheriae*. Every worker in this laboratory has made the observation that guinea-pigs or mice receiving an incubated mixture of a heterologous antitoxin (Type B) together with botulinus toxin (Type A) die more promptly than the controls receiving the toxin alone. Bronfenbrenner furthermore noted that if the toxin is diluted, not in salt solution or distilled water, but directly in normal horse serum or even in ordinary broth, its potency is increased as compared with that of the same toxin diluted in distilled water. In a recent paper Coleman reports on some experiments which have some bearing on this subject. A mixture of filtered botulinus toxin and normal guinea-pig leucocytes incubated for several hours greatly increased in toxic potency. These various observations made with the toxin of *B. diphtheriae* and *B. botulinus* lend considerable support to the hypothesis of Dernby and Walbum, who believe that the proteolytic enzymes of the bacilli are the main causative agents in the processes. This theory as applied to the diphtheria toxin is briefly as follows: The major portion of the toxin is formed, neither intracellularly nor set free from the dead, autolyzed bacteria, but originates from the proteoses of the culture medium. The diphtheria bacilli in undergoing autolysis liberate specific proteolytic enzymes, which split the proteoses and peptones of the medium and of the bacilli to lower degradation products. Some of the primary, intermediary split-products are the toxins. Inasmuch as the major portion of the proteoses is resident in the culture medium, it is reasonable to assume that the toxins have

an exogenous origin and the diphtheria toxin is a true exotoxin. In case the proteolytic degradation process continues the toxins are split and lose thereby their specific properties.

Several facts already presented in the papers of Dozier, Wagner and others leave no doubt that the proteolytic enzymes are intimately connected with the toxin production of *B. botulinus*. However proteoses are probably not necessary for the process. Highly potent toxins are produced in biuret free media. Moreover, the toxins once formed are fairly resistant against the action of proteolytic ferments such as pepsin and trypsin (Van Ermengem, Bronfenbrenner and Schlesinger). Furthermore the autogenous ferments of *B. botulinus* have apparently no effect on the toxin. In this respect the botulinus toxin differs fundamentally from that of *B. diphtheriae*.

The theory of Dernby and Walbum is very attractive and deserves consideration in future studies on the toxin production of *B. botulinus*. Unfortunately a number of conditions, as for example the influence of the H-ion concentration on the toxin production, and the toxicity of ground botulinus bacteria, have not as yet been investigated. However the data presented in this paper lend considerable weight to the views of Dernby and Walbum and confirm the view that the toxin production is intimately connected with the nitrogen metabolism and the proteolytic enzymes of the anaerobe.

SUMMARY

1. *B. botulinus* induces in a 2 per cent solution a slow evolution of gas, and a considerable accumulation of ammonia, amino and volatile acids with little change in the reaction of the medium. The addition of glucose influences the metabolic activity of the organism as indicated by a large and rapid evolution of gas, a slight ammonia production and the development of an acid reaction.

2. The maximum chemical changes which take place in glucose Parke-Davis pepton cultures inoculated with vegetative cultures occur in the first forty-eight hours of incubation. The acidity is probably the most important growth limiting factor. In

plain pepton cultures the greatest chemical changes take place between the forty-eighth and ninety-sixth hours of incubation; the exhaustion of specific food substances is probably responsible for the cessation of growth.

3. The presence of glucose in 2 per cent pepton cultures of *B. botulinus* Type B may inhibit the formation of a highly potent toxin.

4. The addition of gelatin to a pepton solution increases the nutritional properties of the medium. It apparently does not increase the buffer value.

5. The activity of *B. botulinus* in aminoids solution indicates that albumoses and proteoses are not necessary for either growth or toxin production.

6. A comparative study of a number of commercial peptones has revealed variations in their nitrogen fractions. In watery solutions of most of the peptones the characteristic toxin is produced. Peptic-digest liver-broth meets all the requirements of a good bacteriological pepton and furnishes highly potent toxins.

7. In beef heart cultures of both Types A and B very potent toxin may be present after eleven days of incubation. Toxin production is intimately connected with the proteolytic activity of the culture.

8. *B. botulinus* grows in open flasks with a 1-inch layer of meat; the exclusion of oxygen, however, favors the proteolytic activity of the organism.

9. The volatile acid present in a ten-day-old meat culture has been identified as a mixture of valeric, butyric and acetic acid in the proportion of 3:7:2; as a rule volatile acids of higher molecular weight predominate.

10. A comparative analysis of "old stock" and "recently isolated" cultures of *B. botulinus* in meat media and milk indicates that very slight differences exist in their metabolism. The biochemical activities of *B. sporogenes* and *B. histolyticus* are similar to those of *B. botulinus*. *Vibrion septique* causes only a slight degradation in the nitrogen constituents of the medium.

B. tetani grows slowly but produces, on prolonged incubation, profound chemical changes in meat media.

11. Twenty-one hours fermentation of a medium of low nitrogen content by *B. welchii* causes sufficient loss in biologic value to prevent a subsequent vigorous growth of *B. botulinus*. Evidence is presented which shows that fermentation of media by *B. welchii* for the removal of muscle sugar induces considerable nitrogenous changes. The growth-promoting properties of two lots of fermented double strength veal-infusion—1 per cent Difco pepton, as shown by plate counts, appear closely related to the amount of total nitrogen in the media.

12. A greater number of viable organisms is usually present in sugar-free double strength veal-infusion—1 per cent Difco pepton than in the same medium with added glucose. This result is in contrast to that previously reported for a medium composed of equal parts of unfermented veal-infusion and peptic-digest. The gelatinolytic potency of the supernatant fluid from the glucose-containing culture is less than that of the sugar-free culture, but the latter also maintains a higher level of growth.

13. A detailed study, including frequent and successive plate counts, chemical analyses and tests for enzymes, of milk cultures of two strains of *B. botulinus*, Type A, indicate that milk possesses only a moderate biologic value for *B. botulinus*. Milk cultures of *B. botulinus* contain a casein-coagulating, as well as a casein-digesting, enzyme.

14. The addition of 1.25 per cent glucose to milk increases the gas production and the H-ion concentration, and decreases the production of ammonia; but has no effect upon either the accumulation of amino-acid nitrogen or volatile acids.

15. A solution of casein digest having approximately the same total nitrogen content as milk possesses high biologic value for *B. botulinus* and *B. tetani*. The latent period of the *B. tetani* culture was much longer than that of either strain of *B. botulinus*. *B. botulinus* produces a larger quantity of volatile acids of higher molecular weight than *B. tetani*.

REFERENCES

ARMSTRONG, CHAS , STORY R. V., AND SCOTT, ERNEST 1919 Pub. Health Rep., **34**, 2877.

AUBEL, E. 1921 Compt. rend. Acad. d. sc., **173**, 179.

BAINBRIDGE, T. A. 1911 Jour. Hyg., **11**, 341.

BENGTSON, I. A. 1921 U. S. Pub. Health Rep., **36-2**, 1665.

BENGTSON, I. A. 1923 Pub. Health Rep., **38-1**, 8, 340.

BLANC, J. 1921 Thesis Laboratories de Microbie Technique et de Physiologie de L'Institute Pasteur.

BLANCHETIÈRE, A. 1920 Ann. de l'Inst. Pasteur, **34**, 392.

BERTRAND, G., AND THOMAS 1921 Practical Biological Chemistry, Harcourt, Brace and Howe.

BOSWORTH, A. W., ELKINS, M. G., AND BLANCHARD, M. E. 1922 Jour. Infect. Dis., **30**, 357.

BRADLEY, H. C. 1922 Jour. Biol. Chem., **52**, 467.

BRAUN, H., AND CAHN-BRONNER, C. E. 1922 Biochem. Ztschr., **131**, 226.

BRONFENBRENNER, J. J., DE BORD, G. G., AND ORR, P. E. 1921 Soc. Exper. Biol. and Med., **19**, 16.

BRONFENBRENNER, J. J. 1924 Proc. Soc. Exper. Biol. and Med., **21**, 318.

BRONFENBRENNER, J. J., AND SCHLESINGER, M. J. 1924 Jour. Exper. Med., **39**, 509.

BROWN, J. H. 1921 Jour. Bacteriol., **6**, 555.

BROWN, J. H. 1924 Abst. Bacteriol. **8**, 26.

BUNKER, J. W. W. 1919 Jour. Bacteriol., **4**, 379.

BURROWS, M. T., NEYMANN, C. A. 1917 Jour. Exper. Med., **25**, 93.

BUSHNELL, L. D. 1922 Jour. Bacteriol., **7**, 373.

CLARK, W. M. 1915 Jour. Infect. Dis., **17**, 109.

CLARK, W. M., AND LUBS, H. A. 1916 Jour. Bacteriol., **2**, 1, 109, 191.

COLE, S. W., AND ONSLOW, H. 1916 Lancet, **191**, 9.

COLEMAN, G. E. 1923 Jour. Infect. Dis., **33**, 384.

DAKIN, H. D. 1912 Oxidation and Reduction in Animal Body, Biochem. Monograph.

DAVIS, LEWIS 1917 Jour. Lab. and Clin. Med., **3**, 75.

DE BORD, GEORGE G. 1923 Jour. Bacteriol., **8**, 7.

DENIS, W., AND MINOT, A. S. 1919 Jour. Biol. Chem., **38**, 453.

DERNBY, K. G., AND BLANC, J. 1921 Jour. Bacteriol., **6**, 419.

DERNBY, K. G., AND ALLANDER, B. 1921 Biochem. Ztschr., **123-124**, 245.

DERNBY, K. G., AND WALBUM, L. E. 1923 Biochem. Ztschr., **137-138**, 505.

DICKSON, E. C. 1915 Jour. Amer. Med. Assoc., **65**, 492.

DICKSON, E. C. 1919 Arch. Int. Med., **24**, 581.

DORYLAND, C. J. T. 1916 N. D. Agri. Exper. Bull., **116**, 348.

DOUGLAS, S. R., FLEMMING, A., AND COLEBROOK, L. 1917 Lancet, **2**, 530.

DOZIER, C. C., WAGNER, E., AND MEYER, K. F. 1924 Jour. Infect. Dis., **34**, 85.

DOZIER, C. C. 1924 Jour. Infect. Dis., **35**, 105.

DUBOVSKY, B. J., AND MEYER, K. F. 1922 Jour. Infect. Dis., **31**, 501.

DUCLAUX, E. 1895 Ann. de l'Inst. Pasteur, **9**, 265.

DYER, D. C. 1916 Jour. Biol. Chem., **28**, 445.

DZIERZGOWSKI, S., AND REKOWSKI, L. DE 1892 Arch. d. sc. biol., **1**, 167.
EIJKMAN, C. 1901 Centralbl. f. Bakteriol., **29**, 841.
ELSER, W. J., AND HUNTOON, F. M. 1909 Jour. Med. Res., **20**, 377.
VAN ERMENGEM, E. 1897 Ztschr. f. Hyg. u. Infectionskrankh., **26**, 1.
ESTY, J. R., AND MEYER, K. F. 1922 Jour. Infect. Dis., **31**, 650.
FERMI, CLAUDIO 1890 Centralbl. f. Bakteriol., **7**, 469.
FLEXNER, S. 1907 Jour. Exper. Med., **9**, 105.
FOLIN, O., AND MACALLUM, A. B. 1912 Jour. Biol. Chem., **11**, 523.
FOLIN, O., AND MCELLROY, W. S. 1918 Jour. Biol. Chem., **33**, 513.
FOLIN, O., AND DENIS, W. 1918 Jour. Biol. Chem., **33**, 521.
FOLIN, O., AND WU, HSIEN 1919 Jour. Biol. Chem., **38**, 81.
FORSTER, E. B., AND MEYER, K. F. Unpublished.
FOSTER, L. F. 1921 Jour. Bacteriol., **6**, 161.
FOSTER, L. F. 1921 Jour. Bacteriol., **6**, 211.
FRED, E. B., PETERSON, W. H., AND DAVENPORT, A. 1919 Jour. Biol. Chem., **39**, 347.
GATES, F. L., AND OLITSKY, R. K. 1921 Jour. Exper. Med., **33**, 51.
GORE, S. N. 1921 Indian Jour. Med. Res., **8**, 505.
GRAHAM-SMITH, G. S. 1920 Jour. Hyg., **19**, 133.
HALL, WALKER, I. 1918 Brit. Med. Jour., **2**, 398.
HALL, I. C. 1921 Jour. Infect. Dis., **29**, 321.
HALL, I. C. 1922 Jour. Infect. Dis., **30**, 445.
HALL, I. C. 1924 Jour. Bacteriol., **9**, 211.
HALL, I. C., RANDALL, S. B. 1922 Jour. Infect. Dis., **31**, 326.
HEINEMANN, P. G. 1919 Milk, W. B. Saunders Co., Philadelphia.
HELLER, H. H. 1921 Jour. Bacteriol., **6**, 445.
VON HIBLER, E. 1908 Untersuchung über die pathogen anaëroben.
HILLER, ALMA AND VAN SLYKE, D. D. 1922 Jour. Biol. Chem., **53**, 253.
HISS, P. H., ZINSSER, HAND, RUSSELL, FREDERICK F. 1923 Text-book of Bacteriology.
HOLMAN, W. L. 1919 Jour. Bacteriol., **4**, 149.
JONES, H. M. 1914 Jour. Infect. Dis., **15**, 357.
JONES, H. M. 1916 Jour. Infect. Dis., **19**, 33.
JONES, H. M. 1920 Jour. Infect. Dis., **27**, 169.
JORDAN, E. O. 1906 Biological Studies by pupils of Wm. T. Sedgwick, Chicago, University of Chicago Press, p. 124.
JORDAN, E. O. 1921 General Bacteriology, p. 406.
KAHN, MORTON C. 1922 Jour. Med. Res., **43**, 155.
KENDALL, A. I. 1911 Jour. Med. Res., **25**, 117.
KENDALL, A. I., AND WALKER, A. W. 1915 Jour. Infect. Dis., **17**, 442.
KENDALL, A. I. 1921 Chem. Jour. Metallurgical Engineer, **24**, 56.
KENDALL, A. I. 1922 Jour. Infect. Dis., **30**, 211.
KENDALL, A. I., DAY, A. A., AND WALKER, A. W. 1922 Jour. Infect. Dis., **30**, 141.
KENDALL, A. I. 1923 Jour. Infect. Dis., **32**, 362.
KLIGLER, I. J. 1916 Jour. Bacteriol., **1**, 663.
KNORR, M. 1924 Zentralbl. f. d. gesammte Hygiene and ihre Grenzgebiete, **7**, 161 and 241.

LANDSTEINER, KARL, AND LAMPL, HANS 1918 Biochem. Ztschr., **86**, 343.
LLOYD, D. J. 1916 Jour. Path. and Bacteriol., **21**, 113.
LONG, ESMOND R. 1920 Amer. Rev. Tuberc., **5**, 857.
LONG, ESMOND R. 1923 The Chemistry of Tuberculosis by Wells, H. Gideon De Witt, Lydia M. and Long, Esmond R.
MADSEN, THORWALD 1908 Handbuch der Technik and Methodik der Immunitatsforschung, p. 41.
MATZUSCHITA, TEISI 1900 Centralbl. f. Bakteriol., **28**, 303.
MCLEOD, J. W., AND WYON, G. A. 1921 Jour. Path. and Bacteriol., **24**, 203.
Medical Research Committee 1919 Reports of the Committee upon Anaerobic Bact. and Infect. Med. Res. Com. Spec. Rept. Series No. 39.
MEYER, K. F., AND OMAN, K. Paper in preparation.
MORGENROTH, T., AND PANE, D. 1906 Biochem. Ztschr., **1**, 354.
NEVIN, MARY 1921 Jour. Infect. Dis., **28**, 226.
NOGUCHI, HIDEYO 1911 Jour. Exper. Med., **14**, 99.
NORTH, C. E. 1909 Jour. Med. Res., **20** 359.
OMELIANSKY, W. L. 1907 Lafar's Handbuch der Technischen Mykologie, p. 576.
RANDALL, S. B., AND HALL, I. C. 1921 Jour. Infect. Dis., **29** 345.
RAUDNITZ 1909 Sommerfeid's Handbuch der Milchkunde, p. 193.
RETTGER, L. F. 1905 Jour. Med. Res., **13** 79.
RETTGER, L. F. 1908 Jour. Biol. Chem., **4**, 45.
RETTGER, L. F., BERMAN, N., AND STURGES, W. S. 1916 Jour. Bacteriol., **1**, 15.
ROBERTSON, MURIEL 1916 Jour. Path. and Bacteriol., **20**, 327.
ROBERTSON, T. B. 1924 Australian Jour. Exper. Biol. and Med. Science, **1**, 30.
ROBINSON, H. C., AND RETTGER, L. F. 1917 Jour. Med. Res., **36**, 357.
ROBINSON, H. C., AND RETTGER, L. F. 1918 Jour. Bacteriol., **3**, 209.
RONA, R., AND ARNHEIM, F. 1913 Biochem. Ztschr., **48**, 35.
SCHMAILOWITSCH, F. 1903 Ref. in Biochem. Centralbl., **1**, 230.
SCHÜBEL, K. 1923 Arch. f. exper. Path. u. Pharmakol., **95-96**, 193.
SEARS, H. J. 1916 Jour. Infect. Dis., **19**, 105.
SHAFFER, P. A., AND HARTMANN, A. F. 1920-1921 Jour. Biol. Chem., **45**, 349.
SHIPPEN, L. P. 1919 Arch. Int. Med., **23**, 346.
SMITH, THEOBALD 1890 Centralbl. f. Bakteriol. and Parasitenkunde, **7**, 502.
SMITH, THEOBALD 1893 Wilder Quarter Century Book, Ithaca, N. Y. Comstock Pub.
SMITH, THEOBALD 1895 Centralbl. f. Bakteriol. and Parasitenkunde, **18**, 1.
SMITH, THEOBALD 1898 Jour. Boston Soc. Med. Sc., **2**, 236.
SÖRENSEN, S. P. L. 1912 Ergebn. Physiol., **12**, 393.
SPITZER, GEORGE, AND WEETER, H. M. 1917 Proc. Indiana Acad. Sc., Indianapolis, p. 91.
STICKEL, J. E., AND MEYER, K. F. 1918 Jour. Infect. Dis., **23**, 68.
STURGES, W. S., AND RETTGER, L. F. 1922 Jour. Bacteriol., **7**, 551.
TAROZZI, GIULIS 1905 Centralbl. f. Bakteriol., Abt. in Orig., **38**, 619.
THOM, C., EDMONDSON, R. B., AND GILTNER, L. T. 1919 Jour. Amer. Med. Assoc., **73**, 907.
TISSIER, H., AND MARTELLY 1902 Ann. de l'Inst. Pasteur, **16**, 865.

Upson, F. W., Plum, H. M., and Schott, J. E. 1917 Jour. Amer. Chem. Soc., **39**, 731.
Van Slyke, D. D. 1912 Jour. Biol. Chem., **21**, 275.
Van Slyke, D. D., and Cullen, G. E. 1914 Jour. Amer. Med. Assoc., **62**, 1558.
Van Slyke, L. L., and Baker, J. C. 1919 Jour. Biol. Chem., **40**, 345.
Wagner, E. 1924 Jour. Infect. Dis., Jour. Infec. Dis., 35, 353.
Wagner, E., Dozier, C. C., and Meyer, K. F. 1924 Jour. Infect. Dis., **34**, 63.
Waksman, S. A. 1918 Jour. Bacteriol., **3**, 475.
Walbum, L. E. 1909 Ztschr. f. Immunitätsforsch. u. exper. Therap., **3**, 70.
Warden, C. C., Connell, I. T., and Jolly, L. E. 1921 Jour. Bacteriol., **6**, 103.
Wilcox, H. L. 1921 Jour. Bacteriol., **6**, 407.
Wilcox, H. L. 1922 Jour. Infect. Dis., **30**, 536.
Wolf, C. G. L., and Harris, J. E. G. 1917 Jour. Path. and Bacteriol., **21**, 386.
Wolf, C. G. L., and Telfer 1917 Biochem. Jour., **11**, 213.
Wolf, C. G. L., and Harris, J. E. G. 1918 Jour. Path. and Bacteriol., **22**, 1: ibid., **22**, 270.
Wolf, C. G. L. 1920 Brit. Jour. Exper. Path., **1**, 288.
Wolf, C. G. L. 1921 Brit. Jour. Exper. Path., **2**, 266.
Wolf, C. G. L. 1922 Brit. Jour. Exper. Path., **3**, 295.
Worth, M. C. 1920 Jour. Bacteriol., **4**, 603.

CLOSTRIDIUM MULTIFERMENTANS IN CHOCOLATE CREAM CANDIES

GRACE A. HILL[1]

Camp Kearny, California

Received for publication September 15, 1924

The object of the investigations upon which this paper is based, was to try to solve a problem of confectioners—the cracking of chocolate creams.

We are here presenting a description of a saccharolytic anaerobe which we found to be uniformly present in the cracked chocolate creams; with an account of our experiments to determine its rôle in the spoilage.

ISOLATIONS AND IDENTIFICATION

Of 24 samples of spoiled chocolate creams examined, 18 showed the presence of gas-producing organisms. Four of these showed good gas production in the initial tubes, but the gas-producers were not isolated. From 1 sample *C. bifermentans* was isolated; from two, *C. tertium;* and from the remaining 11, a saccharolytic anaerobe which we think to be the *C. multifermentans* of Stoddard (1915).

Where the gas-former was demonstrated but was afterwards lost, and where other gas-formers than *C. multifermentans* were isolated, there was every reason for believing, from morphology, stains and cultural characteristics, that it was present in the initial fermentation tubes, but was lost in the isolation process.

This organism was also isolated three times from "mazetta cream," a commercial preparation used by confectioners as a

[1] The author is indebted to Dr. Ivan C. Hall, with whose valuable council the work was executed, at the University of California, Berkeley, California, August to December, 1922.

foundation for cream fillings; and three times from egg albumin, which is in turn the foundation for mazetta cream. *C. sporogenes* was not met with at any time in our investigations.

All of the isolations, of the supposed species of *C. multifermentans*, from the candies, the egg albumin and the commercial cream, were cultured as a series. No differences were found and they were decided to be of the same species and type.

We are classing this organism as *C. multifermentans*[2] only tentatively. Perhaps it should be considered as a special type of this species, since it does not ferment inulin. This difference however, may be due to technique. Stoddard (1919) does not give his method for making the fermentation tests. Our method was that of Hall (1922). Meat-infusion broth was rendered sugar-free by the action of the Welch bacillus, and the carbohydrate, in solution in distilled water, was sterilized separately, and added just before inoculation.

MORPHOLOGY

The organisms isolated, which were evidently, as will be shown later, those responsible for the splitting of the chocolate coat of cream-filled candies, and which we are considering to be a strain of *C. multifermentans*, are large, though slender, bacilli, occurring singly or in pairs. Filaments are formed in some media—most notably in those lacking sugar.

Gram stain. The organisms retain the Gram stain in young cultures, but quickly lose this property. In glucose broth after eighteen hours incubation there may be as many Gram-positive as Gram-negative individuals, but in twenty-four hours the culture is largely Gram negative. This is also true of glucose-agar cultures. (It is possible, however, that the Gram-negative rods are those that have been inhibited or killed by acid produced in the sugar media. This theory is supported by the fact that within a week glucose cultures are usually found to be

[2] The organism is so named to comply with Bergey's Manual of Determinative Bacteriology. We believe that it is more like the *Clostridium butyricum* of Prazmowski than the organism chosen by the committee as the type species, which is stated not to form the typical clostridia.

dead.) Infusion agar and Petroff's egg-medium cultures, on the other hand, retain the Gram's stain after three days of incubation.

Shadow forms. There is a tendency, even in young cultures, to the production of "shadow forms," which do not retain any stain well. These, especially in sugar media, frequently show deeper staining granules, sometimes one in each end of a pale swollen rod. Forms resembling the well-known *Vibrion septique* "citrons" are also formed.

Starch granules. If dilute iodine is added to a hanging drop made from a sugar medium culture, the rods are found to contain many granules which take a deep purple stain, showing the storing of a starch-like substance.

Spores. Spores are formed in twenty-four hours in brain media, blood agar, Petroff's egg and infusion agar. Spores are produced sparingly in glucose infusion agar shakes, and abundantly on glucose infusion agar plates. Spores have not been observed in any meat-extract, sugar media, although there is abundant growth with gas production.

The spores are rather straight-sided ovals, produced subterminally or, less frequently, in the center of the rod. The bacilli swell in their production to form clubs or spindles. Occasionally a thickened spore-bearing rod has straight sides. The spores appear as refractive bodies with the Gram stain, and are acid-fast. A great variety of forms are found in spore production on some media, most notably Petroff's egg medium.

Spore germination. On infusion-glucose-agar plates spores may apparently be formed, become distended, and germinate within forty-eight hours. Forms have been observed fulfilling all of the stages pictured in Prazmowski's classical illustration of the polar germination of *Clostridium butyricum*. These cultures are Gram negative except for what appear to be spores in the early stages of development within the rods, and also for the small new rods as they leave the old distended spore cases.

Motility. Motility is variable. It is uniformly absent in glucose broth. A few motile individuals may usually be observed in preparations from young brain cultures, especially if some of the solid portions of the medium are carried over to

the slide. The majority of individuals in any field are non-motile.

CULTURAL CHARACTERISTICS

Infusion agar. No growth occurs in extract agar, nor indeed in any medium that is sugar-free. When colonies are produced in infusion agar shakes, they are irregularly lenticular masses. Surface plate colonies on infusion agar, at forty-eight hours, appear as small round transparent dew-drops. The hand-lens reveals irregular edges.

Glucose agar. In glucose-agar stab-cultures, in twenty-four hours, gas is produced that fragments the agar so violently as, not infrequently, to push out the cotton plug. Colonies are knobby and irregularly lenticular. They are commonly broken up by subsequent gas production. In from two to three days gas production fragments the agar.

On a glucose-infusion-agar slant or plate the surface colonies are small, white, opaque and raised. These cultures, however, are never very satisfactory because the agar becomes torn with gas bubbles, and some liquefaction of the media takes place, which overflows the surface.

Fermentation in all sugar media is accompanied by a distinct odor of butyric acid.

Petroff's egg medium. In forty-eight hours colonies are formed abundantly. They are small, averaging 1 mm. in diameter, opaque, yellowish in color, ameboid in shape, and viscid in consistency.

Blood agar. Dew-drop colonies are formed which have irregular edges and are non-haemolytic.

Gelatin. Gelatin is not liquefied, even when sugar is present.

Brain. Gas is produced in brain media, but no blackening takes place.

Broth. No growth takes place in sugar-free broth. In glucose broth 80 per cent or more of gas is produced in twenty-four hours. Even before rapid gas production ceases the organism has a tendency to clump and settle to the bottom of the tube, so that in forty-eight hours the broth is clear. A hanging drop

of a twenty-four-hour, glucose-broth culture usually shows this clumping, so that the preparation has the appearance of an agglutinating Widal. Because of this property immunization-agglutination tests for differentiation could not be used.

Milk. In twenty-four hours there is acid and gas, with a fairly firm clot below the marble seal. At this time a clot may be forming above the seal, or it may not appear for several days. In forty-eight hours the clot below the seal is firmer than at twenty-four hours, and is somewhat riddled with gas bubbles; although it is not so firm nor so shredded as in the typical *C. Welchii* reaction.

Starch agar. These organisms produce a strong diastase. Large halos appear about the colonies on starch agar plates. These are accentuated by the addition of dilute iodine. In sugar-free, 1 per cent starch agar-shake-cultures gas is produced with a clearing of the medium, and after forty-eight hours of vigorous growth the starch is found, by the iodine test, to have disappeared completely.

Fermentation. The organism ferments, with the formation of acid and gas, glucose, lactose, sucrose, raffinose, salicin, glycerol and starch; but not mannitol or inulin.

Pathogenicity. The organism was found to be non-pathogenic for guinea pigs. Eleven of the isolations were inoculated as twenty-four-hour glucose-broth-cultures, intraperitoneally, 1 cc. each, into as many (11) guinea pigs. The pigs were apparently unaffected.

THE SPLITTING OF THE CHOCOLATE COAT

In an attempt to help solve the problem of a local confectioner who was having trouble with the cracking of chocolate creams, we made a cream filling in the laboratory, following his receipt. His "mazetta" was used as a foundation.

Clostridium multifermentans was isolated, without difficulty, from the mazetta used. Also a sample of the egg albumin that had been used in making the mazetta, was found to contain this organism to the number of more than 10,000 per gram.

About 10 cc. of the cream filling made in the laboratory was

put into each of twenty sterile test tubes, and a layer of paraffin was added to simulate the chocolate coat. Ten of these were inoculated with *C. multifermentans*, and ten were left for controls. Within a week gas was produced in eighteen tubes, inoculated and uninoculated alike, breaking or tipping the paraffin coat, with subsequent oozing.

Two tubes, one inoculated, and one control, failed to respond. It was noticed that these had been given paraffin coats of unintentional thickness. After three weeks incubation, on the way from the incubator to the discard, they met with a jarring accident. A few days later they were noticed to have undergone a stormy fermentation, with a lifting of the paraffin coat to the cotton plug. This was undoubtedly made possible by the jar and loosening of the paraffin. This is recorded as support for the theory that a strong chocolate covering may prevent splitting even when other conditions are favorable for gas production.

In this instance a fermentation tube inoculated from the control cream, one month after it had been put into the tube, produced *C. multifermentans* in pure culture.

Fifteen samples of chocolate creams, from as many batches (none of which showed spoilage), were obtained from six different confectioners. When inoculated with *C. multifermentans*, 9 of the 15 cracked characteristically. The organisms inoculated were recovered from the split candies, fulfilling Koch's postulates.

It had previously been observed that apparently good candies, from batches having typically cracked candies, usually were found to contain *C. multifermentans*, although they themselves showed no spoilage. Also the organism was at no time found to be present in samples from batches in which none of the candies showed spoilage. It seemed logical to expect, however, that occasionally *C. multifermentans* might be found in candies from batches which had shown no spoilage; so we took the precaution of inoculating fermentation tubes from the centers of the candies that had been opened, aseptically, to receive the inoculation. None of the samples inoculated were found to contain *C. multifermentans* previous to the inoculation.

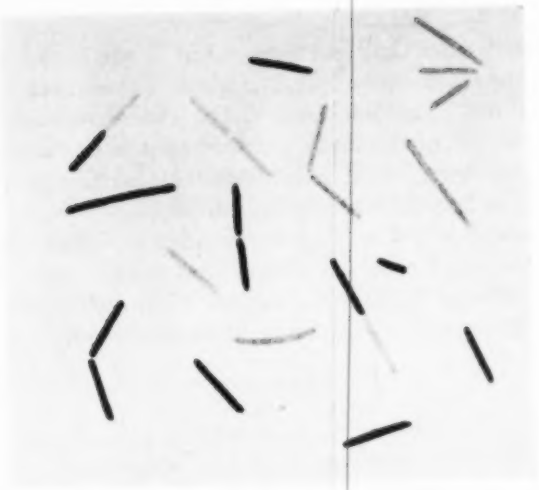


FIG. 1. C. MULTIFERMENTANS. GRAM STAIN. TWENTY-FOUR HOURS IN GLUCOSE BROTH

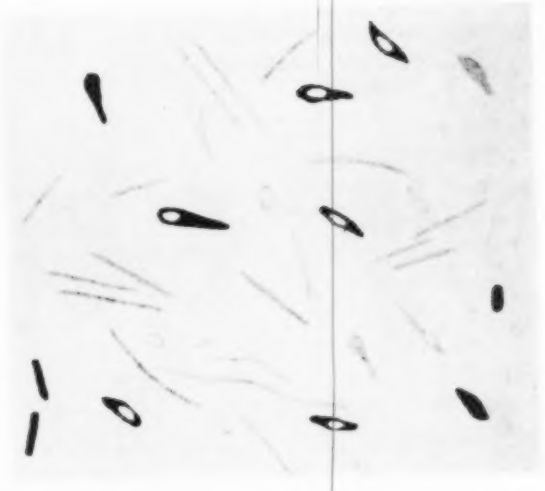


FIG. 2. C. MULTIFERMENTANS. GRAM STAIN. FORTY-EIGHT HOURS ON PETROFF'S EGG MEDIUM

As to the candies that had not reacted (6 out of the 15 inoculated), it appeared there may have been one of several reasons for the failure. The thickness of the chocolate coat, and the moisture content of the filling were thought to be sometimes the controlling factors. Also some fruit-flavored fillings are made so acid as to be undoubtedly inhibitive.

The attempt is not made here to solve in detail the confectioner's problem, but it will appear that safety might lie either in making creams that do not contain *C. multifermentans*, or in rendering them unfavorable for its development.

SUMMARY

In the light of our various investigations it would seem probable that most of the spoilage of chocolate creams through splitting is due to a gas-forming microorganism whose morphology and cultural characteristics correspond to those of *Clostridium multifermentans* (Stoddard).

REFERENCES

HALL, IVAN C. 1915 Univ. of Calif. Publications in Pathology, **2**, 147.
HALL, IVAN C. 1922 Jour. Infec. Dis., **30**, 445.
STODDARD, J. L. 1919 Lancet, **196**, 12.

RELATION OF TEMPERATURE TO THE GROWTH OF THERMOPHILIC BACTERIA

FRED W. TANNER AND GEORGE I. WALLACE

Department of Bacteriology, University of Illinois, Urbana

Received for publication September 30, 1924

The growth function of microörganisms has received considerable study and is known to be influenced by a great many factors, of which temperature is one of the most important. The thermophilic bacteria constitute a group which is especially adapted to a study of the effect of temperature on growth. These organisms have such a high optimum temperature for growth that it is easy to select lower temperatures of incubation and still be within the range for active growth of the mesophilic bacteria.

RELATION OF TEMPERATURE TO GROWTH

Historical

The growth of bacteria was early studied by Buchner, Longard and Riedlin (1887). Using the plate method they calculated the generation time of cultures of *Vibrio cholerae*. Growth was apparently regular since no lag phase was observed. Following this work there was a period in which growth studies were limited to the lag phase. Müller (1895) was probably one of the first investigators to report a lag phase. His work was confirmed later by Hehewerth, Rahn and others. Later Barber (1908) reported his observations on the growth of a single cell of *Bacterium coli*. He found growth to be constant and regular, without a latent period. To obtain such a growth, however, he stated that transplanting must be made from actively growing cultures. Barber's work probably cannot be closely compared with that of other investigators on this subject

because of the difference in technic. Barber used single cells and followed their development microscopically while other investigators used the standard plate. Lane-Claypon (1909) working on *Bacterium coli*, *Bacterium typhosum*, and *Bacterium enteritidis*, found that after the lag phase was over, the growth was regular until a maximum was reached when it became constant for a short period and then slowly decreased. M'Kendrick and Pai (1911) made a mathematical study of the growth of *Bacterium coli* and reported conclusions similar to those of Lane-Claypon. In addition they reported that the rate of multiplication was proportional to the concentration of nutriment in the medium. Penfold and Norris (1912) reported similar observations on *Bacterium coli* and confirmed the work of M'Kendrick and Pai.

The general opinion of some of the early investigators was that growth should be divided into four phases: (1) the lag phase or period of no growth; (2) the period of constant rapid growth; (3) the maximum stationary phase, the period in which the organisms multiply only slowly when they are at the maximum of growth and; (4) the period in which the number of organisms decreases. Buchanan (1918) extended this scheme by dividing growth into seven phases: (1) the initial stationary phase; (2) the lag phase or positive growth acceleration phase; (3) the logarithmic growth phase; (4) the negative growth acceleration phase; (5) the maximum stationary phase; (6) the accelerated death phase, and (7) the logarithmic death phase. His paper did not contain the detailed experimental work upon which these conclusions were based. A very complete study of growth was made by Graham-Smith (1920) with *Staphylococcus aureus* in plain broth. He found that the normal growth was rapid for the first day, became slower on the second, and finally dropped rapidly until a low number of cells was reached, this number remaining constant for a long period. The rapid rise and fall occurred within six or seven days at 37°C. He incubated his cultures at different temperatures and found that the lower the temperature, the longer it took to reach a maximum but that the maximum when reached at a low temperature

was higher than at higher incubation temperatures. This was true until the temperatures became low enough to be inhibitive, when the organisms began to decrease as soon as they were inoculated. His work is a fine addition to our knowledge of the development and growth of bacteria.

In the past few years there have been investigations of growth from a different angle. Henrici (1921, 1924) has studied the growth of *Bacillus megatherium*, *Bacterium coli*, and a diphtheroid bacillus both by the use of a haemocytometer and by biometric study of the cells. He found in all cases that the cells increased in length until they were about six times the length of the inoculated cells. This maximum size was reached shortly after the beginning of the logarithmic growth phase. During this logarithmic growth phase the cells rapidly became shorter. He could not, however, find any correlation between the variations in size and the rate of cell division. Clark and Ruehl (1920) also noticed this change in morphology. They believed that from two to six hours after transplanting, the organisms were twice as large as when inoculated. This shows that growth (increase in cell size) may take place before it is apparent by methods which estimate the number of new cells formed.

Experimental

In the experiments here reported one liter flasks were used containing 600 cc. of plain broth. Inoculations were made from two day old cultures of thermophilic bacilli which had previously been subcultured for twenty-four hours at 55°C. in order to activate them. Glass tumblers with a layer of cotton in the bottom were inverted over the necks of the flasks in place of the usual cotton plugs. As samples had to be removed from the flasks at frequent intervals, this method reduced the possibility of contamination to a minimum and also decreased evaporation. Samples were taken at twenty-four hour intervals and plates were made. Later, when growth was more regular, counts were made every third day. Flasks of each culture were incubated at the three temperatures, 55°, 37°, and 20°C. The growth curves on chart 1 were made from the

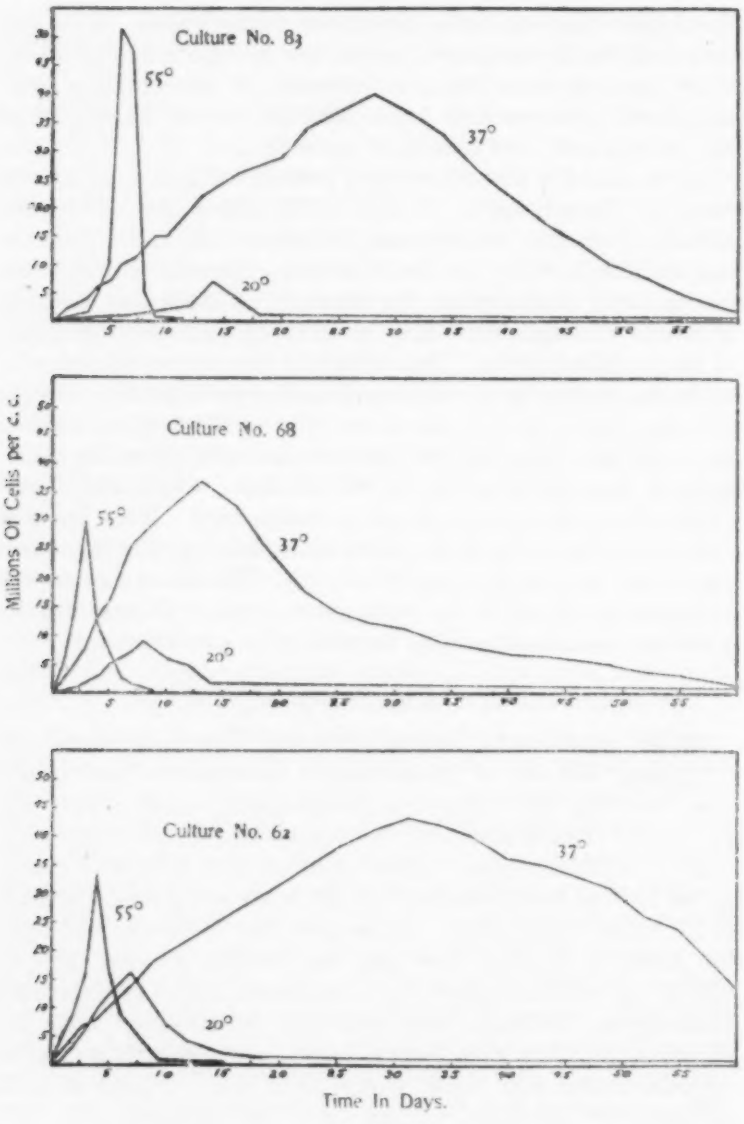

Culture No. 83
55°
37°
20°
Culture No. 68
55°
37°
20°
Culture No. 62
55°
37°
20°
Millions Of Cells per c. c.
Time In Days.

CHART 1

observations of two different series. Both trials were similar so that there were no great variations.

Discussion of results

At 55°C., under the condition outlined above, the three cultures acted as real thermophiles. The maximum growth was reached in a much shorter time than at the other two temperatures. The initial stationary phase of Buchanan was very short except in the case of strain 83 where it seemed to be slightly more protracted. It is interesting to note that at 55°C. the cultures soon died out. This is in accordance with the general understanding that temperature exerts a driving action on cellular activities and that at 55°C. the bacterial cell works much faster and consequently wears itself out much more quickly. This is also brought out by the fact that cultures of thermophilic bacteria die out rapidly at 55°C. and must be frequently transferred if they are to be maintained. Lyman and Langwell (1923) in work on the decomposition of cellulose for the making of acetic acid by thermophilic bacteria stated that they secured 40 times as much product at 55°C. as at 37°C. Inoculations of thermophilic bacteria in the center of a petri dish of sterile agar will give a colony 2 cm. in diameter in ten to twelve hours at 55°C. These facts indicate that the strict thermophiles grow far better at 55°C. than at lower temperatures.

At 37°C. a typical unimodal curve was secured, the high point being reached in different times by the various strains. Maximum growth was secured in about twenty-eight days with strain 83, in about eighteen days with strain 68, and in about thirty-two days with strain 62. With strain 83 the maximum growth at 55° was much higher than the maximum at 37°C. Just the reverse was true with the other two cultures. With these strains, as would be expected, a higher maximum growth was secured at 37°C. but the time was much longer. This was what Graham-Smith found with the mesophile *Staphylococcus aureus*. The lower the temperature of incubation, the greater was the growth. Our curves for 37° and 55°C. with culture

68 are hard to reconcile with the conception of a strict thermophile.

At 20°C. the growth was very different. The maximum of growth was, in each strain, lower than at 55° or 37°C. The highest maximum growth was secured with strain 62 in about eight days. However, in every case, there was a distinct decrease in the number of cells after this period of maximum growth. It is interesting to note that at 20°C. the cells were not all dead even after a long time. The numbers became constant in eighteen days and remained so over a period of six weeks. Counting was stopped at that time. The cold seemed to act as a preservative. The high point reached at 20°C. by culture 62 was somewhat unexpected.

LAG PHASE STUDIES WITH THERMOPHILIC BACTERIA

Historical

Müller (1895) early observed the lag phase. Hehewerth (1901) and Rahn (1906) did similar work on different species, finding that the lag period varied with different organisms. Barber (1908), as before stated, could not find a lag period in his microscopical observations on the rate of growth of a single cell of *Bacterium coli*. Lane-Claypon (1909) found a period during which there was no increase in the number of bacteria present. In 1914 Ledingham and Penfold reported an equation expressing the mathematical analysis of lag. They found a definite phase during which growth proceeded regularly but slowly. This work and that of Barber's conflicted in some ways with that of the other investigators. Penfold (1914) helped to clear up this confusion. His study of the factors influencing the bacterial lag showed that if *Bacterium coli* was subcultured when actively growing, it would continue to grow at the same pace. He also showed that the lag is lengthened when the temperature is lowered and when old cultures are used for inoculation. His work is of particular interest here because of its similarity to the work taken up in the present investigation. Slator (1916) found the lag phase to be a mathematical process

and determined a constant for it. Chesney (1916) confirmed the work of some of the early investigators. He explained the lag phase as an expression of injury. Salter (1919) working on *Bacterium coli* further confirmed the work on this subject and emphasized the influence of age of culture on the lag phase.

The work of Clark and Ruehl (1920) and Henrici (1921–1922–1923) reveals another aspect of the lag phase phenomenon. Their researches indicate that from the standpoint of the cell, there may be no such thing as a lag phase because the cells grew even in what is called the lag phase when measured by cell division (on the standard agar plate). This suggests that the older conception of the term lag rests on cell division and not on growth; because a cell may grow, as shown by Clark and Ruehl, and Henrici, before it divides. In this respect Clark's and Henrici's work confirmed the results of Barber who also used microscopic methods. Henrici reported that cells of *Bacillus magatherium* began to increase during the so-called lag phase. This would seem to suggest that our conception of what is now called the lag phase depends on what definition of growth is assumed and also on the methods for determining it. Clark and Ruehl stated that the most interesting changes took place in a culture during the first 12 hours of growth. Sherman and Albus (1922–1923) also found this morphological difference between new and old cells and their work shows that the presence of salts greatly affect the period of lag. On the assumption that lag is a period of physiological youth or a process of rejuvenescence, they suggested that the conception of young tissue in a physiological sense be applied to the unicellular organisms.

Experimental

One hundred cubic centimeter flasks containing 50 cc. of plain broth were used for this study. Inoculations were made from three-month-old cultures and from actively growing eleven-hour cultures into cold and warm medium. The warm medium was previously warmed to the temperature of incubation for the particular part of the investigation being carried out. The size of the inoculation was also varied, inoculations

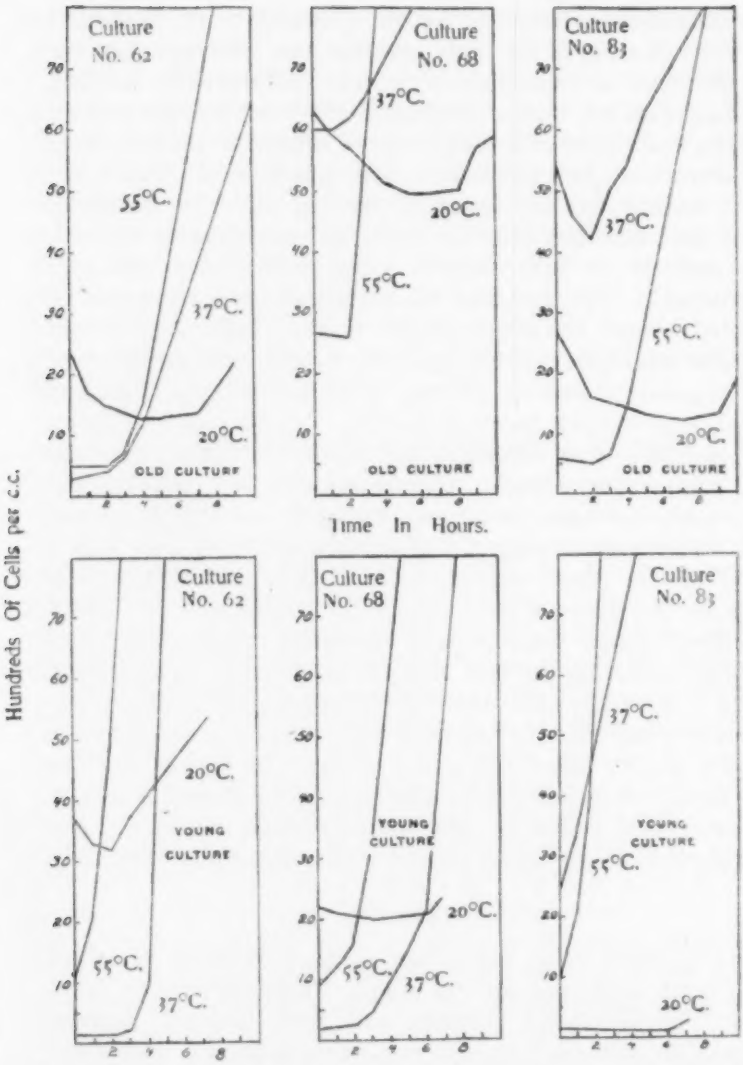


CHART 2. SHOWING THE EFFECT OF AGE ON THE LAG PHASE OF THREE STRAINS OF THERMOPHILIC BACTERIA IN PLAIN BROTH AT 55°, 37°, AND 20°C.
Using one loop for inoculation

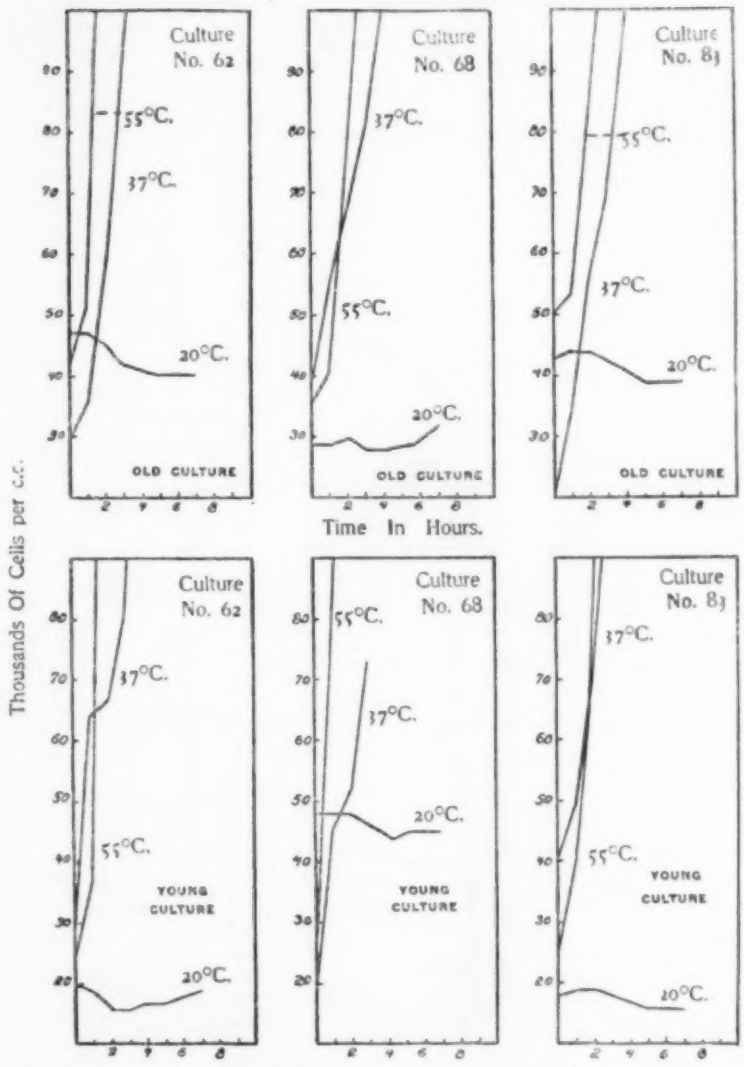


CHART 3. SHOWING THE EFFECT OF AGE ON THE LAG PHASE OF THREE STRAINS OF THERMOPHILIC BACTERIA IN PLAIN BROTH AT 55°, 37°, AND 20°C.
Using 0.1 cc. for inoculation

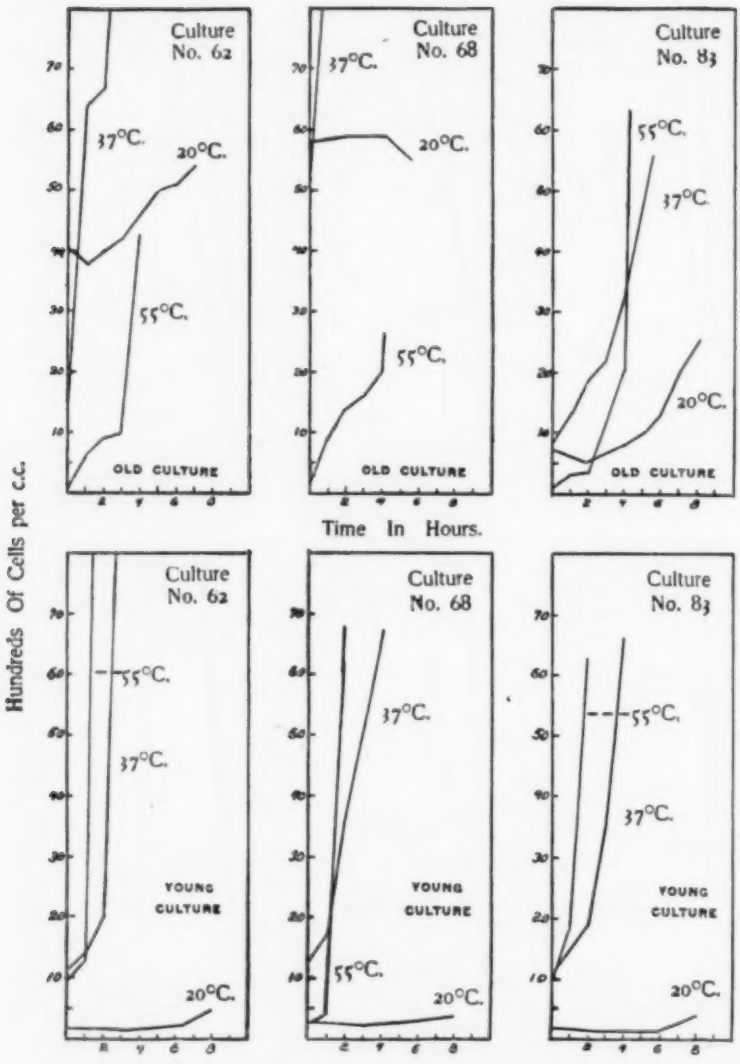


CHART 4. SHOWING THE EFFECT OF AGE ON THE LAG PHASE OF THREE STRAINS OF THERMOPHILIC BACTERIA IN PLAIN BROTH AT 55°, 37°, AND 20°C.
Using one loop for inoculation, and warmed to temperature of incubation

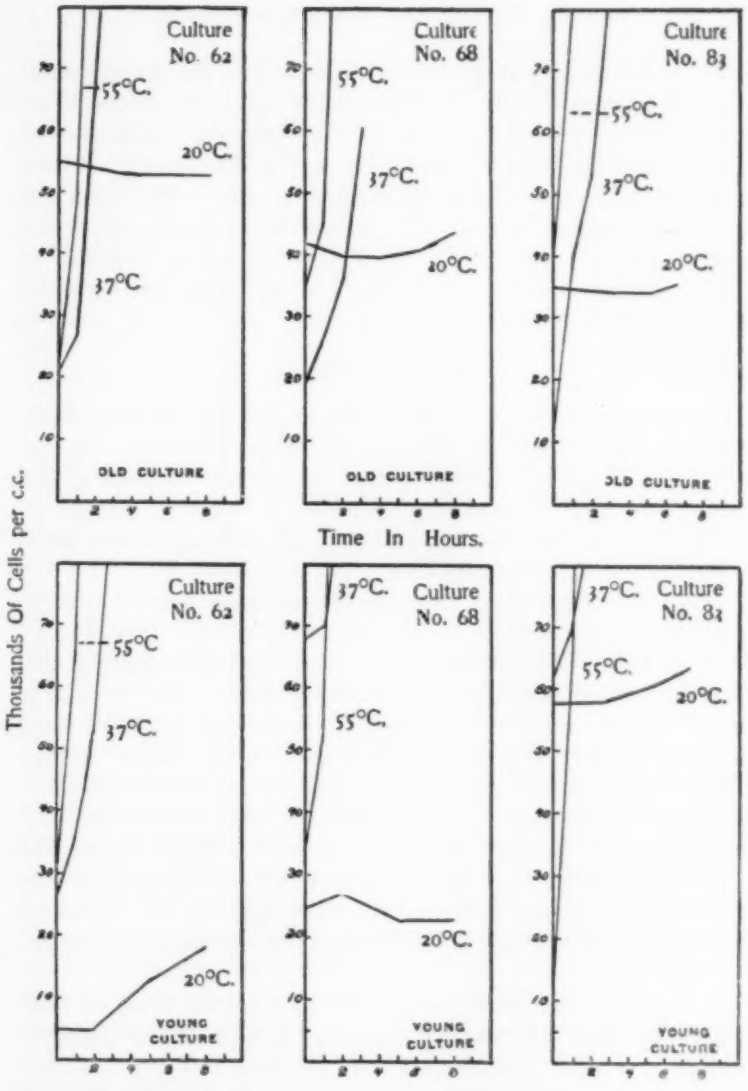


CHART 5. SHOWING THE EFFECT OF AGE ON THE LAG PHASE OF THREE STRAINS OF THERMOPHILIC BACTERIA IN PLAIN BROTH AT 55°, 37°, AND 20°C. Using 0.1 cc. for inoculation, and warmed to temperature of incubation

being made by loop and with a pipette. In the former case, one loop full was used for the inoculation and in the latter 0.1 cc was used. Counts were made at hour intervals until growth as evidenced by turbidity could be seen in the flasks. It was found by trials that when growth could be seen in the flasks the lag phase was over, and that the culture had reached one of the later stages of growth. The results are shown in charts 2, 3, 4, and 5. Duplicate plates were made and it is felt that the curves represent the actual growth rate.

Discussion of results

It has been shown by Penfold and others that the lag phase may be markedly altered by changes in the environment. In this work an attempt was made to show what effect the age of the culture, the size of inoculum, and the initial temperature of the medium has on the lag phase. All of these studies were carried out at three temperatures, 55°, 37°, and 20°C.

In the literature there seems to be no agreement of opinion in regard to just what is meant by the lag phase. Penfold and Chesney both considered it as the period between seeding and the start of maximum growth. Slator stated that it was a period during which bacteria grow at a slower rate than they do later. Lane-Claypon explained lag as the period during which there is no increase in the number of bacteria present. Buchanan said it was the period between the initial stationary phase and the logarithmic growth period. Others have said it was a period during which the number of cells decreased. In our work the lag phase has been looked upon as the period of growth between the time of seeding and the starting of maximum growth.

At 55°C. the lag phase was found to be from three to four hours long when old cultures were inoculated in small amounts (1 loopful) into a medium that had been held at room temperature (chart 2). All three of the cultures acted similarly although culture 83 showed a decided decrease in numbers that was not shown by the others. Its revival was quick, however, and in four hours it was growing rapidly. Culture 68 was in its maxi-

mum growth period in four hours. The growth was stationary or decreased slightly for two hours and then began to increase, slowly at first but becoming faster until the maximum growth period had started. When actively growing young (eleven-hour) cultures were used the lag period was almost entirely eliminated, the logarithmic growth phase starting almost immediately. All three cultures acted very much alike in this respect. There was no stationary period as each culture started growing immediately after being transplanted. At 37° and 20°C. with an old culture an evident lag was secured. This was markedly reduced when young actively growing cultures were used.

If the size of inoculation was increased from one loopful of the old culture to 0.1 cc. of the old culture another shortening of the lag was noticed (chart 3). Again all three of the cultures acted the same. During the first hour there was an increase in the number of cells but it was comparatively small. This was greater in the second hour and logarithmic phase started by the third hour. When young cultures were used for inoculation the logarithmic growth period started immediately. With culture 68 the growth was extremely rapid but with cultures 62 and 83 less so.

An attempt was then made to see if there would be a change in the lag phase if the medium used was heated up to the temperature of incubation before inoculation (charts 4 and 5). Comparing chart 2 with chart 4, and chart 3 with chart 5, it will be seen that in every case the lag was shorter in the medium that had been previously warmed to the temperature of incubation. When young cultures and large inoculations were used the same results were obtained with one exception; culture 68 reached its maximum growth when transplanted into the cold medium slightly earlier than when transplanted into the warm medium. As in both cases maximum growth was reached in an hour this difference was probably not important.

At 37°C. the lag period was longer than at 55°C. When old cultures were inoculated into the cold medium it took from four to six hours for the maximum growth to start (chart 2). The

three cultures formed almost identical curves of growth. The growth remained practically constant for two hours and then slowly began to increase. At 55°C. the increase was faster. When young cultures were used for inoculation the two hour stationary period was the same but the subsequent increase was faster. In culture 83, however, the growth started almost immediately and increased faster than it did with either culture 62 or culture 68.

With the larger inoculations the results were like those at 55°C. (chart 3). There was a shorter lag period, maximum growth starting between the first and second hour after seeding. The growth was not as rapid as at 55°C. When the young cultures were used the results were again comparable to those at 55°C. Maximum growth started after a period of one hour and then the increase was rapid. In every case the three cultures acted very much alike.

The effect upon the lag phase of warming the medium to 37°C. before inoculation was determined at this temperature also. Again, comparing charts 2 and 3 with charts 4 and 5, respectively, it will be seen that in every case the growth was more rapid in the flasks where the medium had been previously warmed to the temperature of incubation than in the flasks where the medium had been held at room temperature before incubation. With two of the cultures, 68 and 83, in the young culture inoculations on chart 5, growth the first hour was slower but afterwards it was faster than in the corresponding trials in the cold medium series. Culture 83 in the young culture cold medium series grew as well as it did when in the warm medium. Growth was rapid in both cases from the start.

In comparing the 55°C. and the 37°C. results, one finds that in all but one case the 55°C. cultures reached maximum growth in a shorter time than when they were incubated at 27°C. When one loop each of the old cultures was inoculated into a medium warmed to 55°C. and incubated at 55°C. the maximum growth did not start until after three or four hours had elapsed; while in the medium heated to 37°C. and incubated at 37°C. growth started rapidly and the maximum was reached in two

or three hours. Perhaps the optimum temperature for incubation was lower than 55°C. and the sudden change from the old culture into the heated medium resulted in an inhibitory action. This reaction was not noticed in the large inoculations, however. It is possible that in the large inoculations there were enough resistant cells to make up for the less resistant ones.

At 20°C. there were many irregularities, as might be expected, because of the temperatures of incubation being so far from the optimum for these organisms. The lag phase in all cases lasted eight hours or more. Plates were not made after eight hours as a comparison of the lag period at different temperatures was all that was wanted. Growth was stationary or decreased from the start. In general, there was no comparable difference when conditions were altered, as there was in the 55° and 37°C. studies. In culture 68 of the large inoculation, young culture warm medium series (chart 5) and in cultures 68 and 83 in the large inoculation old culture cold medium series (chart 3) growth increased slightly during the first two hours but decreased after that. Culture 62 in the small inoculation young culture cold medium series (chart 2) and in the large inoculation young culture warm medium series (chart 5) increased after a two hour stationary period. The same was true of cultures 62 and 83 in the small inoculation old culture warm medium series (chart 3). This growth was not very rapid. The results show that 20°C. is a very unsatisfactory temperature for normal growth of the three cultures. The cells reproduce just enough to keep the cultures alive.

SUMMARY AND CONCLUSIONS

1. Growth curves were prepared for three thermophilic bacteria at 20°, 37°, and 55°C., and in plain broth. At 20°C. the cultures showed less growth than at 37° or 55°C. At 37°C. the period of maximum growth was reached in from sixteen to thirty days. At 55°C. growth was very rapid, the cells dying quickly after the period of active growth. Cells of thermophilic bacteria growing at 55°C. wear themselves out very quickly, the cultures becoming sterile.

2. Warming of the medium to 55°C. before inoculation in every case shortened the lag phase of growth.

3. At 37°C. the lag phase was longer than at 55°C. When both young and old cultures were used, the former began growing much more rapidly. The period of lag was about the same for both young and old cultures.

4. At 20°C. the growth of the thermophiles was quite irregular. The lag phase was greatly prolonged. At 20°C. the bacteria grow slowly; the number soon reached a level where it remained constant for a long period.

REFERENCES

BARBER, M. A. 1908 The rate of multiplication of Bacillus coli at different temperatures. Jour. Inf. Dis., **5**, 379–400.

BUCHANAN, R. E. 1918 Life phases in a bacterial culture. Jour. Inf. Dis., **23**, 109–125.

BUCHNER, LONGARD AND RIEDLIN 1887 Uber die vermehrungsgeschwindigkeit der bakterien. Cent. Bakt., 1 Abt., **2**, 1.

CHESNEY, A. M. 1916 The latent period in the growth of bacteria. Jour. Exper. Med., **24**, 387–418.

CLARK, PAUL, F., AND RUEHL, W. H. 1919 Morphological changes during the growth of bacteria. Jour. Bact., **4**, 615–629.

GRAHAM-SMITH, G. S. 1920 The behavior of bacteria in fluid cultures as indicated by daily estimation of the number of living organisms. Jour. Hyg., **19**, 134–204.

HENRICI, A. T. 1921 A statistical study of the form and growth of a spore bearing bacillus. Proc. Soc. Exper. Biol. Med., **19**, 122–123.

HENRICI, A. T. 1922 A statistical study of the form and growth of a diphtheroid bacillus. Proc. Soc. Exper. Biol. Med., **20**, 179–180.

HENRICI, A. T. 1923 A statistical study of the form and growth of *Bacterium coli*. Proc. Soc. Exper. Biol. Med., **21**, 215–217.

HENRICI, A. T. 1924 A statistical study of the form and growth of *Bacterium coli*, Proc. Soc. Biol. and Med., **21**, 215 and 216.

LANE-CLAYPON, JANET 1909 Multiplication of bacteria and the influence of temperature and some other conditions thereon. Jour. Hyg., **9**, 239–248.

LEDINGHAM, J. C., AND PENFOLD, W. J. 1914 Mathematical analysis of the lag phase in bacterial growth. Jour. Hyg., **14**, 242–260.

LYMAN, A. H., AND LANGWELL, H. 1923 Action of bacteria on cellulosis materials. Jour. Soc. Chem. Ind., **42**, 279–287.

M'KENDRICK, A. G., AND PAI, M. K. 1911 The rate of multiplication of micro-organisms: A mathematical study. Proc. Roy. Soc. Edin., **31**, 649–655.

MÜLLER, M. 1895 Ueber den einfluss von fieber temperaturen auf die wachstumgeschwindigkeit und die virulenz des typhus bacillus. Zeit. Hyg. und Infektionskr., **20**, 245.

PENFOLD, W. J. 1914 On the nature of bacterial lag. Jour. Hyg., **14**, 215–241.

PENFOLD, W. J., AND MORRIS, DOROTHY 1912 The relation of concentration of food supply to the generation time of bacteria. Jour. Hyg., **12**, 527–531.

RAHN, O. 1906 Ueber den einfluss der stoffwechselprodukte auf das wachstum der bakterien. Cent. Bakt., **16**, 417–429, 606–617.

SALTER, R. C. 1919 Observations on the rate of growth of Bacterium coli. Jour. Inf. Dis., **24**, 260–284.

SHERMAN, J. M., AND ALBUS, W. R. 1923 Physiological youth in bacteria. Jour. Bact., 8, 127–139.

SHERMAN, J. M., AND ALBUS, W. R. 1922 Salt effects in bacterial growth. Salt effects in relation to the lag period and velocity of growth. Jour. Bact., **7**, 583–588.

SLATOR, A. 1917 A note on the lag phase in the growth of microorganisms. Jour. Hyg., **16**, 100–108.

HYDROGEN SULPHIDE PRODUCTION BY ANAEROBIC SPORE-BEARING BACTERIA

MORTON CHARLES KAHN

Department of Hygiene, Cornell University Medical College, New York City

Received for publication October 8, 1924

That commercial peptones vary in the amount of hydrogen sulphide which they will yield when incorporated into suitable culture media supporting growth of various species of bacteria capable of elaborating this substance, has been pointed out by Myers (1920), Thompson (1920–1921) and Tilley (1923a). This last named investigator also demonstrated that differences in hydrogen sulphide production may be shown for various strains of bacteria within the same species. In a later communication, Tilley (1923b) reported that while unoxidized sulphur in the form of cystin yielded an abundance of hydrogen sulphide, the unoxidized sulphur of commercial peptones may consist largely of some compound or compounds other than cystin, and not utilizable by bacteria for the production of H_2S.

To obviate this difficulty and to avoid the necessity of testing each lot of pepton individually when hydrogen sulphide tests are to be made, Tilley advocated the addition of known quantities of an unoxidized sulphur in the form of sodium thiosulphate to media containing the usual lead acetate indicator. In this way Tilley succeeded in producing a uniform black coloration of the media when the bacteria planted were able to elaborate hydrogen sulphide, regardless of the initial cystin content of the pepton used.

As no report has heretofore appeared in regard to the hydrogen sulphide producing properties of the anaerobic spore-bearing bacterial group, a study of this subject has been undertaken, and for this purpose I have used the sodium thiosulphate media as advocated by Tilley, but modified somewhat to suit the requirements

of this group of organisms. Instead of using 3 per cent of pepton, 1 per cent was employed and 0.5 per cent of agar instead of 3 per cent, as anaerobes find some difficulty in growing in a very stiff substance. Casein digest fluid (Kahn, 1922) was also added as an enriching agent as some of the anaerobes will not multiply actively in a plain pepton-meat infusion mixture. The formula, then, for the substance used to support growth and test for H_2S in these experiments was as follows: Beef heart infusion, 1000 cc., pepton (Difco) 10 grams, casein digest fluid 30 cc., agar 5 grams, sodium thiosulphate crystals, c.p. (Baker) 2.5 grams. The above ingredients were heated to 100°C. for thirty minutes to dissolve, the reaction adjusted to pH 7.2, filtered through cotton and flannel, tubed in 10 cc. amounts and sterilized in the autoclave at 15 pounds for twenty minutes.

Just prior to inoculating, the tubes were boiled for fifteen minutes to expel as much of the dissolved oxygen as possible, rapidly cooled to 46°C. and 0.1 cc. of a sterilized 10 per cent solution of lead acetate added to each tube. Inoculations were made from 0.5 per cent casein digest agar cultures with the aid of a Pasteur pipette, employing about 0.25 cc. of inoculum. In all experiments with anaerobic spore-bearing organisms definite results are more likely to be obtained within a minimum of time if relatively large amounts of culture are used for transplanting. Often a mere loopful of material when seeded from one tube to another will not be sufficient to bring about a desired growth or reaction until considerable time has elapsed. Anaerobiosis was induced in all cultures by applying a cap of sterile vaseline about $\frac{3}{4}$ inch in height. As has already been reported (Kahn, 1922) this method of inducing growth was found to be an effective and simple one, capable of satisfying the oxygen requirements of the most fastidious species. The tubes were incubated at 37°C. for twenty days and daily observations made during this period.

The following types of spore bearing anaerobes were employed in these experiments, the numbers in parenthesis indicating the number of strains tested: the *Vibrion septique* (3), *B. oedematiens* (*B. novyi*) (2), *B. welchii* (5), *B. fallax* (1), *B. tertius* (1), *B. chauvoei* (1), *B. sphenoides* (1), *B. putrificus* (3), *B. aerofoetidis* (2), *B.*

tetani (2), *B. bifermentans* (3), *B. sporogenes* (3), *B. centrosporogenes* (1), *B. botulinus* (2), *B. histolyticus* (4). These cultures were all offsprings of single bacterial cells isolated by the Barber technic and there would seem to be no question of their purity. Suitable tests for aerobic contamination were made by inoculations on casein digest agar slants. I was unable to detect any such contamination during the investigation.

The species of bacteria capable of elaborating hydrogen sulphide in this medium invariably produced an intense black coloration of the entire substance. This reaction was quite obvious with the positive strains after twenty-four hours and very marked after two days incubation. The organisms seemingly incapable of elaborating H_2S did not produce any alteration in the color of the medium which exhibited a turbid grey appearance due to the addition of the lead acetate.

On the basis of the results shown in table 1, it seems feasible to divide the anaerobic spore-bearers into two groups, i.e., producers and non-producers of hydrogen sulphide. Those anaerobic spore bearers capable of elaborating H_2S were found to be *B. aerofoetidis, B. putrificus, B. tetani, B. bifermentans, B. sporogenes, B. centrosporogenes, B. botulinus, B. sphenoides, B. welchii* and *B. tertius,* while the following were found to be incapable of producing this substance: *B. fallax, B. oedematiens, B. chauvoei* and the *Vibrion septique. B. histolyticus* was the only organism encountered giving what might be termed a borderline reaction. Although 3 strains of this species were investigated and the tests duplicated on several occasions, the reaction about to be described was invariably obtained. After four days incubation the tubes seeded with this organism still resembled the controls in color, leading one to believe that although a strongly proteolytic species, *B. histolyticus* was incapable of elaborating H_2S, whereas with the frankly positive group the jet black coloration was produced after forty-eight hours. On the fifth day, however, the cultures of *B. histolyticus* were found to have taken on a distinct tan hue and after ten days had elapsed this color deepened somewhat, but was hardly comparable to the black coloration produced by the definitely positive types. On the basis of this test it seems

TABLE 1

Hydrogen sulphide production by anaerobic spore-bearing bacteria in lead acetate sodium thiosulphate medium

SPECIES OF ANAEROBE	NUMBER OF STRAINS	24 HOURS	48 HOURS	72 HOURS	4 DAYS	5 DAYS	6 DAYS	10 DAYS	12 DAYS	15 DAYS	20 DAYS
Vibrion septique	3	−	−	−	−	−	−	−	−	−	−
B. oedematiens	2	−	−	−	−	−	−	−	−	−	−
B. chauvoci	1	−	−	−	−	−	−	−	−	−	−
B. fallax	1	−	−	−	−	−	−	−	−	−	−
B. tetani	2	+	++++	++++	++++	++++	++++	++++	++++	++++	++++
B. putrificus	3	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++
B. acrofoetidis	3	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++
B. bifermentans	3	++	++++	++++	++++	++++	++++	++++	++++	++++	++++
B. sporogenes	4	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
B. centrosporogenes	1	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
B. botulinus	3	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
B. histolyticus	3	−	−	−	−	+	++	++	++	++	++
B. welchii	5	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++
B. tertius	1	++	++++	+++	++++	++++	++++	++++	++++	++++	++++
B. sphenoides	1	++	++++	++++	++++	++++	++++	++++	++++	++++	++++
B. bellonensis	1	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++

+ indicates presence of blackening.

TABLE 2

Hydrogen sulphide production by anaerobic spore bearing bacteria in lead acetate peptone agar. No sodium thiosulphate added

SPECIES OF ANAEROBE	NUMBER OF STRAINS	24 HOURS	48 HOURS	72 HOURS	4 DAYS	5 DAYS	6 DAYS	10 DAYS	12 DAYS	15 DAYS	20 DAYS
Vibrion septique	3	−	−	−	−	−	−	−	−	−	−
B. oedematiens	2	−	−	−	−	−	−	±	±	±	±
B. chauvoci	1	−	−	−	−	−	−	−	−	−	−
B. fallax	1	−	−	−	−	−	−	−	−	−	−
B. tetani	2	++	++	++	++	++	++	+++	+++	+++	+++
B. putrificus	3	++	++	++	++	++	++	++	+++	+++	+++
B. aerofoetidis	3	++	++	++	++	++	++	++	++	++	++
B. bifermentans	3	−	+	+	++	++	++	+++	+++	+++	+++
B. sporogenes	4	++	+++	+++	+++	++++	++++	++++	++++	++++	++++
B. centrosporogenes	1	++	+++	+++	+++	+++	+++	+++	++++	++++	+++++
B. botulinus	3	+	++	+++	+++	+++	+++	+++	+++	++++	++++
B. histolyticus	3	−	−	−	−	−	−	−	−	−	−
B. welchii	5	−	+	+	+	+	++	++	++	++	+++
B. tertius	1	−	+	+	+	+	+	+	++	++	++
B. sphenoides	1	+	+	+	++	++	++	++	++	++	++
B. bellonensis	1	+	++	++	++	++	++	++	++	++	++

+ indicates presence of blackening.

that *B. histolyticus* is extremely slow and weak in its H_2S elaborating properties but that there is some activity in this regard cannot well be disputed.

A second series of experiments was undertaken to test the hydrogen sulphide activities of this group of bacteria in a medium containing lead acetate but where no unoxidized sulphur was added in the form of sodium thiosulphate. The conditions of this experiment were otherwise identical with the first. The results are reported in table 2. It will be noticed that the result, where one depends on the inherent cystin content of the pepton as a mother substance of H_2S, is less distinct and takes a considerably longer time to become manifest than where sodium thiosulphate is added. The degree of blackening is not as intense and also is produced in such varying degree that it would obviously tend to make more difficult the interpretation of the test. The reactions, also, lagged somewhat as compared with those taking place in the medium to which sodium thiosulphate had been added. *B. histolyticus* failed to darken this type of substance at all, suggesting that unoxydized sulphur in the form of sodium thiosulphate is more available than cystin for this species as a source of hydrogen sulphide. These several findings establish the value of adding sodium thiosulphate to a medium when tests for hydrogen sulphide are made with members of this group.

Uninoculated controls were carried with each of the above experiments and exhibited no change in coloration during the observation period.

DISCUSSION

For years observers have considered the ability or lack of ability of certain anaerobic spore bearers to blacken animal tissue when incorporated into suitable culture media, as a cultural characteristic of differential importance. This phenomenon has been variously explained. Rettger (1906) considered the blackening due to hydrogen sulphide production. Von Hibler (1908), according to Hall and Petterson (1924), correctly attributed the blackening to the formation of iron sulphide in meat or brain

mediums by sulphuretted hydrogen set free from the protein molecule. Henry (1917) thought that this discoloration may have been due to a tyrosine derivative brought about by the interaction of tyrosine and tyrosinase, or a humin-like substance due to the condensing of sugar with an amino body. Hall and Peterson (1924) have recently published a painstaking investigation on the possible mechanism of this phenomenon and have arrived at the conclusion that it is due to the iron content of brain medium, when such is used alone, or to the iron content of pepton, when this substance is added. They were able to demonstrate that iron, as it is found in various brands of commercial pepton, varies widely in the amount present, Difco pepton being most constant in this regard. When iron was added to their media, containing various brands of pepton, the following variations with the same species were found as regards the time taken to produce blackening: *B. welchii*, two to five days; *B. novyi* (*B. oedematiens*) two to five days; *B. tetani*, one to five days. When no iron was added to the basic substance containing various brands of pepton, even wider variation was encountered with some of the organisms, for example: *B. sporogenes*, two to four days, *B. histolyticus*, two to twenty-two days, *B. tyrosinogenes*, two to seven days, *B. welchii*, seven days, several of the brands of pepton not giving rise to any blackening with *B. welchii* after forty-five days and with *B. tetani* after one to thirty days. Some of the brands showed no blackening with *B. tetani* after forty-five days.

Thus we see that wide variations are liable to occur when recording the blackening phenomenon produced by various members of the anaerobic spore bearing group. Such discrepancies would seriously handicap accurate observation of this important cultural characteristic, depending on the brand of pepton used, when readings are taken solely from meat or brain mash with or without the addition of iron. The modified medium of Tilley, as described here, was found to yield uniform results as regards the detection of the elaboration of hydrogen sulphide by these bacteria. Rapid and constant blackening took place where a positive reaction occurred, making the test clear-cut and readily

interpreted; while on the other hand where no H_2S was liberated the medium remained uncolored as did the uninoculated controls.

In view of the variations liable to be encountered in the cystin content of various peptones used in the preparation of media, as reported by Tilley, or of the iron content, as demonstrated by Hall and Peterson, and in the possible variation of the iron content of natural brain and muscle tissue, where no pepton is added, it would seem advisable to substitute a medium more accurate in its composition for the recording of this blackening phenomenon and for this purpose we would like to recommend the modified medium of Tilley, as described here.

It is interesting to note that hydrogen sulphide production may not necessarily be an indicator of proteolysis from the bacteriological point of view, as evidenced by the reduction in volume of native protein, for *B. welchii*, *B. tertius* and *B. sphenoides*, non-proteolytic species of anaerobes, are able to elaborate H_2S quickly and strongly as judged by this test, while *B. histolyticus*, an active digestor of native protein, produces H_2S very slowly and in small amount.

CONCLUSION

The medium of Tilley, containing sodium thiosulphate and lead acetate, modified for culturing anaerobic spore bearing bacteria was found to be satisfactory for testing the hydrogen sulphide elaborating ability of this group. Sixteen different species (36 strains) were used in these tests.

There was found to be no essential correlation between the native protein digesting ability and the H_2S producing properties of these organisms.

It is suggested that Tilley's medium be employed in testing the so-called blackening ability of the anaerobic spore bearers instead of the less delicate and indefinite meat mash and brain preparations.

REFERENCES

HALL AND PETERSON 1924 The discoloration of brain medium by anaerobic bacteria. Jour. Bact, **9**, 21.

HENRY 1917 An investigation of the cultural reactions of certain anaerobes found in wounds. Jour. Path. and Bact., **21**, 344.

KAHN 1922 A cultural study of anaerobic spore bearing bacteria with strains isolated by the Barber single cell technic. Jour. Med. Res., **48**, 155.

MYERS 1920 The production of hydrogen sulphide by bacteria. Jour. Bact., **5**, 231.

RETTGER 1906 Studies on putrefaction. Jour. Biol. Chem., **2**, 71.

THOMPSON 1920–21 The group of hydrogen sulphide producing bacteria. Jour. Med. Res., **42**, 383.

TILLEY 1923a Variations in hydrogen sulphide production by bacteria. Jour. Bact., **8**, 115.

TILLEY 1923b The relation between chemical compositions of peptones and hydrogen sulphide production by bacteria. Jour. Bact., **8**, 287.

VON HIBLER 1908 Untersuchungen uber pathogenen Anaeroben, Fisher, Jena.

FURTHER CULTURAL CHARACTERISTICS DIFFERENTIATING B. CHAUVOEI FROM OTHER ANAEROBES

A. S. SCHLINGMAN AND A. W. HAINES

Medical Research Laboratories, Parke, Davis and Company, Detroit, Michigan

Received for publication October 13, 1924

The disagreement among many workers as to the exact cultural characteristics of *B. chauvoei* has existed, to a certain extent, since Bollinger and Feser showed that the disease, blackleg, was to be differentiated from anthrax from an etiological, as well as from a clinical, point of view.

Of late years this organism has been most commonly confused with two other spore-bearing anaerobic bacilli, the *Vibrion septique* and *B. edematis-maligni*.

In a previous paper by one of us (Schlingman, 1924), it was shown by immunization tests that these three organisms were separate and distinct, and that blackleg immunizing agents made from pure cultures of *B. chauvoei* gave no protection against the other two organisms.

The desire to differentiate these organisms further led to the following experiments designed to determine, if possible, some marked characteristic in culture media which would be of aid in further distinguishing one species from the other.

DESCRIPTION OF ORGANISMS USED

Throughout this entire work five strains of *B. chauvoei*, three of the *Vibrion septique* and three of *B. edematis-maligni* were used.

As a basis for description all the strains of these organisms were planted in Hibler medium (Goss, Barbarin and Haines, 1921), liver agar (2 per cent) and 2 per cent glucose agar, deep.

B. chauvoei: All strains of this organism in Hibler medium

produce gas and a slight cloudiness of the liquid which later clears up, as autoagglutination of the organisms takes place. No odor is present except a very faint indication of butyric acid. The brain tissue turns only slightly pink and is not digested. The colonies in liver agar (2 per cent) are small, translucent, and ellipitical in shape. After twenty-four hours incubation little or no gas is produced in deep tubes of this medium. This, however, to a certain extent, seems to depend on the freshness of the medium as in a very fresh medium there may be sufficient gas production to break the agar into small sections. No growth occurs in 2 per cent glucose agar, deep, unless bits of brain tissue or blood are carried over in the seeding.

Vibrion septique: In Hibler medium, after twenty-four hours incubation, this organism produces only a very slight cloudiness of the liquid and usually a rather violent gas production. The brain tissue is not proteolysed, nor is there any odor. In liver-agar (2 per cent), this bacillus forms round, white, wooly colonies with early and extensive gas production, the agar being shattered after twenty-four hours. Colonies of the same character are formed in agar medium containing 2 per cent glucose, with moderate gas formation.

B. edematis-maligni: The three strains of this organism were similar in their action on practically all the media used. They were, however, somewhat different in colony formation and in their activity. In Hibler medium all strains produced darkening of the liquid, proteolysis of the meat pieces, and a fetid odor. The strains designated "Novy" and "Koch" produced no blackening of the tissue except occasionally and then only after prolonged growth (four to five months). The strain designated "Weinberg" usually produced some discoloration of the brain tissue after twenty-four to forty-eight hours incubation. The colonies of this strain in liver and 2 per cent glucose agar are rather large and loosely fluffy, the agar usually being broken by gas production after twenty-four hours growth. The colonies of the Novy strain, the most active of the three, in liver and 2 per cent glucose agar are small, more compact than those formed by the Weinberg strain, and have short fluffy tendrils.

The "Koch" strain, the least active of the three, produced in liver and glucose media, colonies somewhat smaller than those formed by the Weinberg strain but similar in shape, producing a relatively small amount of gas in their growth.

In an effort to find a solid medium for shake cultures, or a liquid medium for fermentation tube cultures, best suited to the needs of these three organisms, practically all of the body tissues and fluids of the ox were used.

The different kinds of media that were made and tried out are given herewith:

1. Liver agar
2. Hormone agar
3. Spleen agar
4. Kidney agar
5. Embryonic muscle agar
6. Amniotic agar
7. Brain agar
8. Glucose agar
9. Glucose serum agar
10. Hibler medium
11. Synthetic medium (agar)
12. Litmus milk (under oil)
13. Litmus milk (under paraffin)
14. Egg-meat medium.

1a. Liver broth
2a. Hormone broth
3a. Spleen broth
4a. Kidney broth
5a. Embryonic muscle broth
6a. Amniotic broth
7a. Brain broth
8a. Glucose broth
9a. Glucose serum broth
10a. Hibler broth
11a. Synthetic medium (fluid)
12a. Litmus milk (fermentations)

All of the above media except the Amniotic, Hibler and Synthetic, were made according to the same formula, as follows: 1000 cc. of water were added to 500 grams of the tissue and cooked in flowing steam for one hour. The resultant broth was strained off and divided into two parts. To one part was added 1 per cent pepton (Witte's) and 0.5 per cent sodium chloride. To the other neither pepton nor salt were added.

These two parts were then heated in flowing steam until the pepton dissolved when each was again divided into two. The reaction of one part containing pepton and one part containing no pepton was adjusted at pH 8.2. The reaction of the remaining portions was adjusted at pH 7.6.

Sufficient broth was then taken from each lot to fill 35 fermentation tubes, the remainder being made into solid medium by the addition of 2 per cent agar. These parts were then heated

in flowing steam until the agar dissolved, filtered, tubed and autoclaved at 15 pounds pressure for twenty minutes.

The fermentation tubes were sterilized by heating in flowing steam for thirty minutes on three consecutive days.

The amniotic medium was made following the outline above except that the amniotic fluid was used as a base, no water being added; the Hibler medium was made according to the usual formula (Goss, Barbarin and Haines, 1921), except as to standardization and lack of pepton and salt; the Hibler broth was made as outlined for other media except that one part of brain infusion was mixed with two parts liver infusion; the synthetic medium (Robertson and Davis, 1923) was a combination of chemicals and contained no meat extract; the Hormone medium was a meat infusion with the addition of 1 egg, 5 grams KH_2PO_4 and 50 cc. glycerol to 1000 cc.; the glucose serum medium was made by increasing the volume of the regular 2 per cent glucose medium with approximately 20 per cent of fresh filtered beef serum; the litmus milk was made as usual, filled into tubes and fermentations, and sterilized by intermittent heating in flowing steam; the egg-meat medium (Rettger, 1906) was a meat-piece medium containing egg-albumin.

Six different lots of each kind of media, except litmus milk and the egg-meat, were made. Only two lots of these latter were used. Five tubes of each lot of the solid media were planted with each strain of the three organisms, the agar being melted and cooled to 45°C. before planting with one loop from the starter cultures in Hibler medium. Three fermentation tubes of the broth in each lot were planted with each strain of the three organisms leaving one tube in each lot unplanted as a control. Three lots of media (liquid and solid) were planted at one time and were incubated at 37°C. for forty-eight hours. Observations of all cultures were made at the end of twenty-four hours and forty-eight hours except those in litmus milk under oil, paraffin and in fermentation tubes which were observed daily for eleven days, as were the cultures in egg-meat medium. The oil and melted paraffin were added to the tubes of litmus milk after planting. As a check against each of the various media, each strain of the

three organisms was planted in the regular Hibler medium and incubated along with the cultures in the special media, these cultures being used as starters for the next planting.

RESULTS OF OBSERVATIONS

In making the various solid media the agar content was the same as that in the media used at the beginning of the work for a basis of description. As a result the growth, when growth did occur, was characterized by formation of the colonies typical for the organism planted, and sometimes gas formation as described in liver agar. Growth of these organisms in fermentation tubes was characterized by cloudiness of the liquid and gas formation or by cloudiness only, depending on the activity of the organisms in a particular medium. The changes which took place in the litmus milk will be described later.

1. *Liver medium:* In this medium all the organisms were very active, especially in those lots containing pepton and salt. In those containing no pepton and salt there was a rather marked decrease in the activity of all strains. In most instances the medium containing pepton was entirely shattered in twenty-four hours while in the other lots but a few bubbles of gas were formed.

All the organisms were active in the fluid form of this medium, but not so much so as in the solid form. In those lots where the final hydrogen-ion concentration was above pH 7.6 *B. chauvoei* either did not grow or its activity was very slight.

2. *Hormone medium:* In the solid medium *B. chauvoei* was not especially active, producing in most instances only a slight amount of gas after forty-eight hours incubation; in the liquid form, a cloudiness of the liquid and in some cases gas after forty-eight hours incubation. The other two organisms were rather active in this medium, both in the solid and liquid, producing gas at the end of twenty-four hours incubation.

3. *Spleen medium:* All the organisms were rather active in this medium except *B. chauvoei* which produced only a slight amount of gas in the liquid after forty-eight hours incubation.

4. *Kidney medium:* Of the three organisms *B. chauvoei* was the

TABLE 1

Results in various media

	B. CHAUVOEI				VIBRION SEPTIQUE				B. EDEMATIS-MALIGNI			
	Pep-ton		No pep-ton		Pep-ton		No pep-ton		Pep-ton		No pep-ton	
	pH 8.2	pH 7.6	pH 8.2	pH 7.6	pH 8.2	pH 7.6	pH 8.2	pH 7.6	pH 8.2	pH 7.6	pH 8.2	pH 7.6
1. Liver agar	+	+	+	+	+	+	+	+	+	+	+	+
Liver broth	+	+	+	+	+	+	+	+	+	+	+	+
2. Hormone agar	+	+	+	+	+	+	+	+	+	+	+	+
Hormone broth	+	+	+	+	+	+	+	+	+	+	+	+
3. Spleen agar	+	+	+	+	+	+	+	+	+	+	+	+
Spleen broth	+	+	+	+	+	+	+	+	+	+	+	+
4. Kidney agar	+	+	+	+	+	+	+	+	+	+	+	+
Kidney broth	+	+	+	+	+	+	+	+	+	+	+	+
5. Embryonic muscle agar*	+	+	+	+	+	+	+	+	+	+	+	+
Embryonic muscle broth*	+	+	+	+	+	+	+	+	+	+	+	+
6. Amniotic agar*	+	+	−	−	+	+	+	+	+	+	+	+
Amniotic broth*	+	+	−	−	+	+	+	+	+	+	+	+
7. Brain agar	+	+	+	+	+	+	+	+	+	+	+	+
Brain broth	−	−	−	−	+	+	+	+	+	+	+	+
8. Glucose agar (2 per cent)	−	−	−	−	+	+	+	+	+	+	+	+
Glucose broth (2 per cent)	−	−	−	−	+	+	+	+	+	+	+	+
9. Glucose serum agar	+	+	+	+	+	+	+	+	+	+	+	+
Glucose serum broth	+	+	+	+	+	+	+	+	+	+	+	+
10. Hibler medium	+	+	+	+	+	+	+	+	+	+	+	+
Hibler broth	+	+	+	+	+	+	+	+	+	+	+	+
11. Synthetic medium (solid)	−	−	−	−	−	−	−	−	−	−	−	−
Synthetic medium (liquid)	−	−	−	−	−	−	−	−	−	−	−	−

	B. CHAUVOEI	VIBRION SEPTIQUE	B. EDEMATIS-MALIGNI
12. Litmus milk (oil)	−	+	+
13. Litmus milk (paraffin)	−	+	+
14. Litmus milk (ferment)	−	+	+
15. Egg-meat medium	+	+	+

\+ = growth.

− = no growth.

* Growth in this medium was not constant.

least active in this medium, although it did grow, producing only a slight amount of gas. The other two organisms were fairly active. None seemed to be affected in their growth by the lack of pepton and salt.

5. Embryonic muscle medium: The growth of *B. chauvoei* in the few instances in which it did occur was characterized by fine elliptical colonies barely visible to the naked eye. In only three tubes of the liquid medium was there any growth noticeable and then only after forty-eight hours incubation. The other two organisms were fairly active, producing a rather moderate amount of gas in most instances, in both the solid and liquid forms.

6. Amniotic medium: The growth of *B. chauvoei* in both the solid and liquid forms of this medium was not constant as it occurred only occasionally and then only in those lots which contained pepton and salt. The *Vibrion septique* and *B. edematis-maligni* grew rather actively, the activity being lessened somewhat by the lack of pepton and salt.

7. Brain medium: B. chauvoei grew with moderate activity in the solid form but did not grow in the liquid in fermentation tubes. The other two organisms were active in both the agar and broth.

8. Glucose medium: No growth of *B. chauvoei* was obtained in any of the lots of either the solid or the liquid forms. The *Vibrion septique* and *B. edematis-maligni* were both very active in all lots of both forms, especially the former, which produced sufficient gas in twenty-four hours in the solid form to shatter the agar completely.

9. Two per cent glucose serum agar: When the 2 per cent glucose medium was enriched by approximately 20 per cent fresh filtered beef serum, *B. chauvoei* grew with a fair degree of activity, the growth being influenced somewhat by the lack of pepton and salt. The other two organisms were very active in both forms of the medium.

10. Hibler medium: All the organisms grew very actively in this medium, the growth being influenced only very slightly, if at all, by the lack of pepton and salt. In the liquid form *B. chauvoei* was the least active of all, showing little or no growth

in those lots where the final hydrogen-ion concentration was above pH 7.6.

11. Synthetic medium: None of the organisms grew in any of the various lots of liquid or solid synthetic medium.

12. Litmus milk under oil: One-half of the tubes in each lot were inoculated by loop, the other half being inoculated by pipet, carrying over at least 0.1 cc. of the liquid from the starter culture. After eleven days observation no growth of *B. chauvoei*, whether inoculated by loop or pipet, has occurred. The *Vibrion septique* inoculated by loop or pipet produces acid and a soft clot in twenty-four to forty-eight hours. After eight or ten days incubation the clot contracts, being entirely surrounded by the whey. No digestion of the clot takes place. *B. edematis-maligni* produces in three to five days acid and a solid clot. Digestion of the clot begins shortly after, it being practically all digested by the tenth or eleventh day.

13. Litmus milk under paraffin: The same procedure was followed as in the litmus milk under oil with the same results.

14. Litmus milk in fermentation tubes: B. chauvoei did not grow in this medium in the fermentation tubes, whether inoculated by loop or by pipet. As in the milk under oil or paraffin there was, in a few instances, where a relatively large amount of the starter culture had been carried over, a slight reduction of the litmus. Transplants from these tubes to suitable media and to other tubes of litmus milk gave negative results.

In the closed arm of the fermentations the *Vibrion septique* produced a solid clot while in the open arm a softer clot was formed. Acid was produced as in the milk under oil and paraffin and also a contracting of the clot.

After three to five days incubation a solid clot was formed by the growth of the malignant edema organisms, this clot turning acid and being almost entirely digested in ten days.

15. Egg-meat medium: All the organisms grew actively in this medium. After eleven days incubation no noticeable changes were evident in the cultures of *B. chauvoei* except active gas production and a faint indication of butyric acid. Those of the *Vibrion septique* showed active gas production with a very slight

pinkening of the meat-pieces. No digestion of meat or albumen took place. In the cultures of *B. edematis-maligni* all of the egg-albumen was digested after five days growth. About three-fourths of the meat pieces were digested by the Novy and Weinberg strains at the end of the eleventh day while during the same time less than one-half had been digested by the Koch strain.

Through the entire experiment it was found that in those media in which growth of the three organisms did occur, the strains of the *Vibrion septique* were by far the most active.

It was found that all the organisms grew over a wide range of H-ion concentration, *B. chauvoei* being influenced more by a high alkalinity than the other two organisms. Best growth was obtained, however, where the final reaction of the medium was between pH 6.8 and 7.6.

SUMMARY

1. Comparisons of the growth of *B. chauvoei*, the *Vibrion septique* and *B. edematis-maligni* were made, using practically all the body tissues and fluids of the ox as a base for both solid and liquid media.

2. *B. chauvoei* does not grow in 2 per cent agar medium containing 2 per cent glucose, but it does grow in this medium when it is enriched by approximately 20 per cent fresh filtered beef serum. Both the *Vibrion septique* and *B. edematis-maligni* are active in the glucose medium and the glucose serum medium.

3. In amniotic medium containing no pepton or salt, *B. chauvoei* did not grow. The other two organisms grew in this medium but their activity was lessened by the lack of the pepton and salt.

4. Both the *Vibrion septique* and *B. edematis-maligni* grew in both the solid and liquid forms of the brain medium while *B. chauvoei* grew only in the solid form.

5. In litmus milk under oil, under paraffin and in fermentation tubes whether inoculated by loop or by pipet, no growth of *B. chauvoei* occurred. The *Vibrion septique*, in this medium, produces acid and a soft clot, no digestion of the clot occurring even after ten days incubation. *B. edematis-maligni* produces acid and a

solid clot which is practically all digested after ten days or eleven days incubation.

6. In egg-meat medium *B. chauvoei* grew as it does in Hibler medium, the only changes occurring being those of gas production with a slight indication of butyric acid. The *Vibrion septique* grew in this medium with active gas production and a slight pinkening of the meat pieces. No digestion of either the meat-pieces or albumen occurred. The growth of *B. edematis-maligni* in this medium was characterized by gas production, darkening of the liquid and, after about five days incubation, a digestion of approximately all of the egg-albumen. After ten to eleven days incubation, one-half to three-fourths of the meat pieces were digested.

7. The cultural characteristics described above may be used in further differentiating *B. chauvoei*, the *Vibrion septique* and *B. edematis-maligni*.

REFERENCES

Goss, Barbarin and Haines 1921 Jour. Inf. Dis., **29**, 615.
Rettger 1906 Jour. Biol. Chem., **2**, 71.
Robertson and Davis 1923 Jour. Inf. Dis., **32**, 153.
Schlingman 1924 Jour. Amer. Veterin. Med. Assoc., **18-6**, 712.

INDOL STUDIES

WALTER L. KULP

From the Laboratory of General Bacteriology, Yale University[1]

Received for publication October 16, 1924

The production of indol by bacteria has received considerable attention throughout the development of the science of bacteriology.

Norton and Sawyer (1921) claimed that, with the exception of *Vibrio cholerae*, all species which have been reported as indol-positive have also been found by some to be indol-negative. Because of this variation among strains of the same species, the indol reaction *per se* cannot be considered as a good species differentiating test. Nicolle and others (1919) studied a number of strains of *Coccobacillus acridiorum*, probably all from the same source, which showed considerable variation in indol formation. Many laboratory workers in bacteriology have met with indol-negative strains of *Bact. coli* and indol-positive strains of *Bact. aerogenes*.

However, because indol production by any given strain of bacterium appears to be constant and fixed (Neisser, 1921), this reaction has some value in the study of individual strains, although an apparent flaw in this argument is brought out in the work of Fabry (1922) who stated that continued cultivation of an indol-positive strain of *Bact. coli* in a medium containing 0.05 per cent phenol developed a strain which did not produce indol. In this instance might not the phenol, by inhibiting growth of the predominating indol-positive cells, bring into prominence an indol-negative bio-type originally present in the same strain? Granting Neisser's claims, the production of indol by bacteria is of some practical value.

[1] The writer wishes to express his thanks to Prof. L. F. Rettger for suggestions and correction of the manuscript.

From experience in this laboratory, and from the conflicting reports of other workers, the author feels that, while individual strains may vary, some of the confusion among writers is due to differences in the technic involved in studying indol production. It is apparent that, in order to get consistent results in the same laboratory and in different laboratories, standard technique should be employed.

The work reported in this paper has been carried out with the hope of adding to our knowledge of the mechanism of indol production, and of providing some standard method or methods which appear warranted as the result of the study and practical application of various methods developed by other workers.

The present work may be conveniently divided as follows:

I. A study of various methods of indol testing
II. A comparative study of media
III. A study of other factors involved in the production of indol by bacteria
IV. The practical application of methods in routine identification work

I. METHODS OF TESTING INDOL PRODUCTION BY BACTERIA

Holman and Gonzales (1923) gave a good historical review of indol testing methods and brought to light an old test which had fallen into obscurity, although Holman has used it since 1911. This is the Gnezda oxalic acid paper test, a method which takes advantage of the extremely volatile nature of indol. Filter paper is saturated with 10 per cent oxalic acid solution and dried. It is cut into strips (as nearly aseptically as possible) and a strip supported across the lower end of the plug in the mouth of the test tube containing the test solution. Indol volatilizes at ordinary room temperature and above, and reacts with the oxalic acid paper, imparting a distinct pink color to it. According to the report of these investigators, this test is specific for indol and checks up well with the Ehrlich-Böhme test. It is very sensitive, a one to one million solution of indol giving a positive reaction in twenty-four hours at 37°C. The paper may be placed in the culture tube when inoculated, and the first development of indol

by the test organism noted. The paper must be kept dry in order to react.

Salkowski's test has gradually been placed in the discard as a test for indol, because various workers have shown that other substances give the same reaction as indol does.

The Böhme-Ehrlich test (para-dimethyl-amido-benzaldehyde and potassium persulfate) has generally been accepted as the standard test by investigators, although there are a few who claim that it is not always specific. The preponderance of evidence, however, is in support of its specificity. This test can not be used with solid or with highly colored media Goré has modified it, however, in such a manner as to make it practical for such media. The test is made by wetting the cotton plug with the given reagents and volatilizing the indol by immersing the culture tube or flask in a boiling water bath. The volatilized indol imparts a characteristic cherry red color to the moistened plug. This modification makes the test very sensitive, one part of indol in two million giving a positive reaction, according to Goré. The Böhme-Ehrlich method has been reported as giving a positive test in 1:300,000 dilution.

In all of the work reported in this paper, the Gnezda, Goré and Böhme-Ehrlich tests have been used simultaneously.

II. MEDIA FOR INDOL PRODUCTION

The medium for indol production generally given in text-books is a pepton broth. This is not always satisfactory. Indol is a decomposition product of the tryptophane molecule. Frieber (1921) assumes that tryptophane is attacked by both positive and negative indol formers with the production of indol-acetic acid, positive organism breaking this up further to indol. Neisser (1921) states that indol is the product of carbon metabolism and not protein metabolism. All bacteria can utilize the amino nitrogen in the alanin part of the tryptophane molecule, but only indol-positive organisms can use the carbon part, leaving free indol. He was not able to prove that indol-positives could break down indol-acetic acid to indol. Neisser also states that tryptophane must be uncombined with protein before indol can be formed from

it by bacteria. Some peptones do not contain free tryptophane, and in others the tryptophane is apparently destroyed by the method of manufacture. Consequently such peptones should not be used for the study of indol production.

Some bacteria are reported to be able to split off tryptophane from the protein molecule, and then to further decompose this tryptophane to indol.

A number of media other than pepton have been suggested by various investigators. Distaso (1917) recommended a pancreatic digest of beef or sheep serum. Pringsheim (1918) inoculated a pepton medium with *B. mesentericus*, incubated the culture for five days, filtered and used the sterile filtrate as a test medium for indol production. Norton and Sawyer (1921) suggest Cannon's hydrolyzed casein as a good medium for indol formation. The author (1921) has noted good indol production in casein digest medium. Zipfel (1912) made a synthetic medium containing pure tryptophane and reported excellent indol production.

An experiment was carried out to determine what simply prepared medium fulfilled the requirements for indol production by indol-positive organisms. The following nitrogen complexes were employed:

1. Tryptic digestion products of Pfanstiehl's C.P. casein
2. Tryptic digestion products of Hammarsten's C.P. casein
3. Tryptic digestion products of Merck's commercial casein
4. Tryptic digestion products of Klim (skim milk powder)

(These were prepared by the method given by the author (1924))

5. Difco pepton
6. Difco pepton plus 0.01 per cent tryptophane
7. Fairchild's pepton
8. Fairchild's pepton plus 0.01 per cent tryptophane
9. Witte's pepton
10. Witte's pepton plus 0.01 per cent tryptophane
11. Difco "tryptophane digest"

Ten grams of each pepton or digestion product, or the equivalent, were employed for each liter of medium. A salt mixture[2] was substituted for meat extract. The reaction was adjusted to pH. 6.8 to 7.0 and sterilization was effected by autoclaving for fifteen minutes at 15 pounds extra pressure.

Four tubes of each medium were inoculated with the test organism. Indol production was noted after forty-eight hours incubation at 37°C. Sterile controls were run by all methods on each medium. Young cultures of three indol-positive *Bact. coli* strains, one indol-positive *Bact. aerogenes*, one indol-negative *Bact. coli* and one indol-negative *Bact. aerogenes* were employed as the test organisms.

Results of this experiment may be summarized as follows:

1. All controls were indol-negative by all tests.
2. The indol-negatives were negative in all media by all tests.
3. Gnezda's, Böhme-Ehrlich's and Gore's tests agreed in all cases.
4. Media 1, 2, 3, 6, 8, 10 and 11 gave strong indol-positive tests by all methods with indol-positive organisms.
5. Medium 7 gave a strong positive with indol-positives except one strain of *Bact. coli*.
6. Medium 4 was negative by all tests.
7. Media 5 and 9 gave negative or only weak positive tests with all positive organisms.

This experiment was repeated in part, employing only media 1, 6, 8 and 11. Nine indol-positive strains of *Bact. coli*, one indol-positive *Bact. cloacae*, one indol-positive *Bact. aerogenes*, two indol-negative strains of *Bact. aerogenes* and one indol-negative *Bact. coli* were employed as the test organisms. Results were entirely in accord with those of the first experiment.

From the results of these experiments, it is apparent that pepton plus tryptophane, casein digest or any medium containing free

[2] $NaCl$ ..... 5.0 gram
$MgSO_4$ ..... 0.2 gram
$CaCl_2$ ..... 0.1 gram
K_2HPO_4 ..... 1.0 gram
} per liter of medium.

tryptophane which will support the growth of the test organism can be employed for indol formation. All of the media which gave good indol formation gave positive tests for free tryptophane. There was no sugar present in any of these media except in number 4. The salt mixture appeared to be a very good substitute for beef extract and had the advantage of being standardized.

III. OTHER FACTORS INFLUENCING THE PRODUCTION OF INDOL BY BACTERIA

Wyeth (1919) states that certain sugars inhibit the proteolytic activity of *Bact. coli* and therefore interfere with indol production. Two per cent glucose stopped indol formation completely; 2 per cent lactose and maltose almost completely; 2 per cent sucrose and mannitol only partially, and 2 per cent starch not at all. Appelmans (1921) confirms these results, reporting that fermentable sugars inhibited or completely stopped indol production by *Bact. coli* and *Proteus vulgaris*. *Bact. dysenteriae* produced indol in the presence of all sugars except glucose, and *V. cholerae* formed indol in the presence of all sugars.

In this work an experiment was carried out to determine the amounts of glucose, sucrose and lactose that were necessary to inhibit or stop indol production. Two media were employed, a casein digest medium and a medium made with Difco pepton plus 0.01 per cent tryptophane. The sugars were sterilized in water solution and added aseptically to the broths in the following percentages: 0.5, 0.25, 0.1, 0.05, 0.025 and 0.01 per cent.

Results

In forty-eight hours an indol-positive strain of *Bact. aerogenes* gave strong indol tests in both media containing glucose up to 0.1 per cent. In seventy-two hours the 0.25 per cent glucose media were positive. After one week there was no positive indol test in either medium containing 0.5 per cent or more of glucose.

It required more glucose to affect the indol production of a strain of *Bact. coli;* 0.25 per cent glucose media showed strong

indol in forty-eight hours and 0.5 per cent in seventy-two hours, but 1 per cent glucose media were negative after one week.

Five-tenths per cent glucose completely stopped indol formation by a strain of *V. cholerae*.

Two strains of *Bact. coli*, one of *Bact. aerogenes* and one of *V. cholerae* gave a strong positive indol test in media containing 1 per cent sucrose after forty-eight hours incubation at 37°C.

The results of both experiments are in agreement with those of Frieber (1921) who stated that indol was not produced in the presence of easily used carbohydrates. However, small amounts do not appear to stop indol production where other constituents of the test medium permit vigorous growth of the organism. In other words, if the easily used carbohydrate is present in small amounts, so that it can be entirely used up by the organism without the metabolic products interfering with its growth, then it can attack any tryptophane present with the resultant production of indol. The time factor must be taken into consideration of course.

Zdansky (1922) observed that indol formation is most rapid at the optimum pH for growth of the test organism. Wyeth (1919) stated that indol production is retarded by free acid or alkali. Barthel (1921) had difficulty in using Zipfel's tryptophane medium and discovered that this was due to the reaction. His results were best when the sterilized medium had a reaction of pH 6.3.

An experiment was carried out to determine the effect of various chemical reactions upon indol production. Casein digest broths were prepared having the following reactions; pH 5.0 to pH 9.0 with intervals of 0.5 on the pH scale.

These media were inoculated with five strains of *Bact. coli*, two of *V. cholerae*, one of *Bact. cloacae*, one of *V. Metchnikovi* and one of *Bact. aerogenes*, all indol-positive strains.

Wherever apparent growth was secured, strong reactions were obtained in forty-eight hour cultures. Therefore, it appears that the pH of the medium affects indol formation only as it affects the growth of the organism.

It has been suggested to the author that perhaps indol-negative

bacteria do produce indol, but that they use it up as quickly as it is formed. This point was studied in the following manner.

Five media were used:

1. Salt mixture
2. Salt mixture plus 0.1 per cent glucose
3. Salt mixture plus 0.1 per cent Difco pepton
4. Salt mixture plus 0.1 per cent casein digest

} plus indol

5. Salt mixture plus 0.1 per cent casein digest

Indol was added to each of the first four solutions so that there was a final concentration of one part of indol to 25,000 parts medium. This amount of indol gives a strong indol test by the accepted methods.

The test organisms employed consisted of five indol-positive strains of *Bact. coli*, one indol-positive *Bact. aerogenes*, one strain each of indol-positive *V. cholerae*, *Bact. dysenteriae* and *Proteus vulgaris*, two strains of indol-negative *Bact. coli*, three strains of indol-negative *Bact. aerogenes*, indol-negative *Bact. paratyphosum*, B., *Staph. aureus* and *Staph. albus*.

Results

1. There was no reduction of indol or apparent growth of any of the test organisms in media 1 and 2.

2. There was no reduction of indol in media 3 and 4, although growth was heavy in every case.

3. All of the indol positives gave a strongly positive indol test in medium 5. On the other hand the indol-negatives proved to be negative.

An attempt was made to induce indol production by continued sub-culturing of indol-negatives in a medium rich in tryptophane, but this was unsuccessful.

The action of a number of organisms, some indol-positive and others indol-negative, was studied in media in which tryptophane

was the only source of nitrogen. The media employed were prepared as follows:

1. Salt mixture plus 0.1 per cent tryptophane
2. Salt mixture plus 0.1 per cent tryptophane plus 0.1 per cent glucose
3. A control medium made up with casein digest

For inoculum, 0.1 cc. of a saline suspension of the test organism was used.

All cultures were incubated at 37° for four days and tested for indol. The organisms employed which had been found to be indol-positive by previous tests in casein digest and tryptophane-Difco media gave 4 plus indol tests in media 1 and 3. Growth was heavy in 3 but questionable in 1. Growth was more in evidence in medium 2 than in medium 1, but no stronger than a one plus indol test was found to be given by any organism in medium 2. Three cultures of *Bact. aerogenes* which had been indol-negative in casein digest or Difco-tryptophane media, gave slight indol production in medium 2, where they showed positive growth. *B. paratyphosum* B was the only other organism to show visible growth in medium 2, but this culture gave no test for indol. Jones and Zinninger (1921) stated that staphylococci grew in media with tryptophane as the only source of nitrogen, but that no indol was produced.

It is apparent that a very slight action of indol-positive organisms upon tryptophane is sufficient for indol liberation. On the other hand, an ever greater growth by indol-negatives in the same medium is not accompanied by the formation of indol. This is rather in accord with the theory of Neisser (1921). The point requires further investigation.

A number of strains of various species were studied in regard to their ability to produce tryptophane or indol or both from a medium containing complex nitrogenous substances but giving no test for free tryptophane.

The sole source of nitrogen in the test media consisted of three different samples of casein and Difco and Whitte's peptones. The casein was dissolved (2 grams to 500 cc. plus salt mixture) in alkali and adjusted to pH 6.8.

These media were inoculated with the following organisms:

B. megatherium
Ps. fluorescens var. non-liq.
Ps. fluorescens var. liq.
E. prodigiosus
Bact. coli
Bact. aerogenes
Bact. dysenteriae
Bact. typhosum
Proteus vulgaris
Staph. albus
Staph. aureus
Bact. paratyphosum A and B
V. cholerae

After a week of incubation at the optimum temperature for each particular species, some of the medium was withdrawn and tested for indol. The remainder of each culture was placed in tubes, 5 cc. to a tube, and sterilized. It was then inoculated with an indol-positive *Bact. coli*. *V. cholerae* was the only organism that was able to produce indol in all five media.

E. prodigiosus, *Ps. fluorescens* var. liq. and *B. megatherium* broke down the casein so that *Bact. coli* produced small amounts of indol in the digested casein medium.

The Difco pepton medium used here gave a positive indol test before inoculation with an indol-positive organism; i.e., indol was present as an impurity. However, the intensity of the reaction increased after incubation with an indol-positive strain of *Bact. coli* for five days at 37°C.

Indol-positives, with the exception of *V. cholerae*, apparently are not able to break down the complex casein with the splitting off of indol. With a comparatively long period of incubation, indol-positives can break down some peptones, solutions of which give no test for free tryptophane, with the splitting off of indol.

On the other hand, some species elaborating a protease, break down the protein, casein, to a less complex nitrogenous substance which does not give the test for free tryptophane but which can be acted upon by indol-positives with the production of indol.

IV. PRACTICAL APPLICATION OF METHODS STUDIED

About 40 different species comprising a total of over 100 cultures were studied in regard to their property of producing indol in a suitable indol-testing medium.

Five media were employed:

1. Casein digest
2. Difco pepton plus 0.01 per cent tryptophane
3. Fairchild's pepton plus 0.01 per cent tryptophane
4. Difco "Tryptophane" digest
5. Zipfel's medium (Barthel, Chr., 1921)[3]

Each culture was run in duplicate in each medium. The Gnezda, Gorè and Böhme-Ehrlich tests were used with each culture.

When a culture showed good growth in forty-eight hours, the indol test was made at that time. When growth was rather scanty, seventy-two or ninety-six hour cultures were employed.

In general, it appears that, in using any of the 5 media employed in this experiment, if an organism is going to produce indol from the tryptophane present, the test will be strongly positive within forty-eight hours or at the appearance of good growth. It does not seem necessary to carry the incubation period any longer. A number of indol-negatives were tested at intervals up to three weeks incubation, with no evidence of indol production.

[3] Zipfel's medium =

	per cent	
Asparagin	0.5	
Ammonium lactate	0.5	
KH_2PO_4	0.2	adjusted to pH 6.8
$MgSO_4$	0.02	
Tryptophane	0.03	

The Gram-negative organisms employed in this study consisted of different strains of the following species:

5 of *Bact. aerogenes*
15 of *Bact. coli*
2 of *Bact. dysenteriae*
3 of *Bact. typhosum*
2 of *V. cholerae*
3 of *proteus*
1 of *V. Metchnikovi*
2 of *Ps. fluorescens*
9 species of Paratyphoids (32 strains in all)

The Gram-positive species consisted of members of the *B. subtilis* group, and various species of cocci.

No Gram-positive species were found to produce indol. Frieber (1921) stated that no Gram-positive bacteria were known to produce indol. However, Morrison and Tanner (1922), in a study of 52 strains of Gram-positive thermophiles, reported that the majority of these produced indol.

Of the Gram-negatives, none of the typhoids or paratyphoids studied produced any indol. All of the Proteus forms, the Vibrios and *Bact. dysenteriae* were indol-positive. Twelve of the *Bact. coli* and two of the *Bact. aerogenes* strains were positive.

All five media gave the same results throughout, and the three methods of testing for indol checked up in every instance.

SUMMARY

1. Suggestions are given for a standardized procedure in testing for indol production by bacteria.

2. The Böhme-Ehrlich, Goré and Gnezda tests are apparently satisfactory.

3. A good medium for indol production by bacteria should contain free tryptophane.

4. It does not appear that indol-negative organisms use indol; neither can they be induced to produce indol by continued subculturing in a tryptophane medium.

5. Satisfactory hydrogen ion concentration for growth of a

bacterial species will also prove favorable for indol production by that species.

6. Media containing small amounts of utilizable carbohydrates and sufficient buffer substance may be employed in testing for indol production by bacteria. However, indol production will be delayed in proportion to the amount of carbohydrate present.

REFERENCES

APPELMANS, R. 1921 Influence des sucres sur la production d'indol. Compt. rend. Soc. de. Biol., **85**, 725–726.

BARTHEL, CHR. 1921 Note on the indol test in tryptophane solution. Jour. Bact., **6**, 85–88.

DISTASO, A. 1917 La formation de l'indol dans le milieu sérum digéré et dilue. Compt. rend. Soc. de Biol., **80**, 253–254.

FABRY, PAUL. 1922 Note sur le bacille coli modifié, ne praduisant plus d'indol. Compt. rend. Soc. de Biol., **87**, 113–115.

FRIEBER, WALTHER 1921 Beiträge zur Frage des Indolbildung und der Indolreaction sowie zur Kenntniss des Verhaltens indol negativer Bakterien. Cent. f. Bakt., 1 Abt., Orig., **87**, 254–276.

GORÉ 1921 Quoted from Holman and Gonzales, 1923.

HOLMAN, W. L., AND GONZALES, F. L. 1923 A test for indol based on the oxalic acid reaction of Gnezda. Jour. Bact., **8**, 577–583.

JONES, S. BAYNE, AND ZINNINGER, PAULINE 1921 The decomposition of tryptophane by Staphylococci without the production of indol. Abst. Bact., **5**, 3.

KULP, W. L. 1921 Casein digest media for growing *L. acidophilus* and *L. bulgaricus*. Abst. Bact., **7**, 9.

KULP, W. L., AND RETTGER, L. F. 1924 A comparative study of *L. acidophilus* and *L. bulgaricus*. Jour. Bact., **9**, 357–394.

MORRISON, L. E., AND TANNER, F. W. 1922 Studies on thermophilic bacteria. Jour. Bact., **7**, 343–366.

NEISSER, M. 1921 Ueber Indol-und Phenolbildung durch Bakterien. Münch Med. Wch., **68**, 1384–1385.

NICOLLE, CH., BLANC, G., ET CAILLON, L. 1919 Sur la valeur de la reaction de l'indol. Compt. rend. Soc. de Biol., **82**, 1126.

NORTON, J. F., AND SAWYER, M. V. 1921 Indol production by bacteria. Jour. Bact., **6**, 471–478.

PRINGSHEIM, E. 1918 Zur Verbilligung und Verschärfung der Indolreaktion. Cent. f. Bakt., 1 Abt., Orig., **82**, 318–320.

WYETH, F. J. S. 1919 The effects of acids, alkalis and sugars on the growth and indol formation of *B. coli*. Biochem. Jour. **13**, 10–24.

ZDANSKY, E. 1922 Untersuchungen über den Stoffwechsel der Bakterien. I. Die Bedentung freier Aminosäuren demonstriert an der Indolreaktion. Cent. f. Bakt., 1 Abt., Orig., **89**, 1–3.

ZIPFEL, HUGO 1912 Zur kenntnis der indolreaktion. Cent. f. Bakt., 1 Abt., Orig., 65–81.

ON BACTERIAL VARIATION

J. ØRSKOV AND ARND LARSEN

State Serum Institute, Copenhagen, Director, Dr. T. Madsen

Received for publication November 8, 1924

During the course of time a number of works have been written on the subject of bacterial variation, and most bacteriologists have now and then met with the experience that a given bacterial culture has behaved in a manner different to that usually seen. Interest in this subject has become more active during recent years, because it has become clear to many bacteriologists that these variations are of considerable importance, both practically and theoretically. The discovery of d'Herelle's virus has certainly contributed a great deal towards this view, as it has become a common occurrence for bacteriologists who have worked with this agent to see radical variations arise in the bacteria they are handling.

It would lead too far to touch upon the literature on this subject; we will therefore simply give a brief description of the observations we have made in the examination of a paradysentery culture belonging to the Group III as defined by Sonne at this Institute as this organism shows a very marked inclination to vary, and in other respects is of interest, especially with regard to the d'Herelle phenomenon just referred to.

The culture in question was a laboratory culture of medium age. A pure cultivation was first made by a method devised by one of us, and only after this treatment was it subjected to examination. With regard to the method itself it need only be mentioned that to a very great extent we have made use of direct microscopical examination of the culture on agar in Petri dishes and have followed the development of the various colonies from their commencement, until growth ceased. This method often gives information which can with difficulty be obtained

even with the most exact macroscopic observation and yields much more certain knowledge as to whether one's variants are actually pure.

On examination of this culture we found that by cultivation on ordinary substrata the culture divided itself into different variants, which on their part were more or less constant.[1]

On an agar dish was spread a stab culture in extract bouillon agar, about fourteen days old, which had been kept in the thermostat at 37° for twenty-four hours and the remainder of the time at room temperature; it was seen, very clearly through the microscope, that there arose two kinds of colonies; some were almost round and fairly sharply contoured, while others grew more diffusely with irregular, winding edges. The various elements in the first-named kind of colony, which we will call "V," proved to be short, stumpy bacilli, while the elements in the last-named were longer and extremely polymorphic; and, what struck one at once was the very early decay in the center of the colonies. The bacteria here were granulated and many cells, even in 12 hours' old colonies, were quite degenerated. Finally, after several days' growth, the central decay in the colonies became macroscopically observable in the appearance of fairly large holes, which involuntarily directed one's thoughts towards the question of "Bacteriophage." We will later on return to the question of bacteriolysis in this variant, which we will call "B." In the V colony too there appeared bacteriolysis, which is a fairly common phenomenon with the paradysentery bacillus, but very much later than in the B colony and much less prominently.

Subcultivations were now made from the two different colony forms V and B, and it was found that the newly formed colonies all resembled those from which they were sown, and when frequent resowing was performed, both colony forms kept quite constant on the surface of ordinary bouillon pepton agar through numerous subcultures.

We now commenced to examine the properties of these two

[1] We have purposely avoided the term "mutations" as this appears to us to be misleading as far as bacterial variation is concerned.

colony forms more closely. In bouillon it was seen that they grew very differently. V always grew diffusely in the first 24 hours, whereafter there appeared a coarse fluffy precipitation; whereas B kept to the bottom of the bouillon, while the liquid above it remained practically clear. The sediment was easily shaken up, but always settled quickly again.

By daily spreadings on agar from the bouillon cultures of V and B which were kept constantly at 37°, it was seen that after a few days both again divided themselves into two, the original and a varying form.

As far as V was concerned, there appeared in the agar spreadings after a couple of days' growth at 37° two kinds of colonies, those which resembled the original V colonies and others which were much more dense and microscopically consisted of somewhat shorter forms than the elements in the V colonies. The boundary between the two colony forms when lying close to each other was always quite well defined, which by the way is a general rule with variations in bacterial colonies, and it was almost always easy to find places in the colonies where one could definitely divide them one from the other, a problem which is naturally, especially here, of capital importance.

With B, too, it was seen that after a couple of days' growth in the bouillon at 37° and subsequent spreading on the agar surface there appeared two kinds of colonies, normal B colonies, and those which resembled them microscopically but did not present the same polymorphy and did not decay like the original B colonies. On cultivating the two new variants in bouillon it proved that in the bouillon they grew differently to both V and B. We will call the form separated from V "M," and that separated from B "Bu." M grew right from the commencement with large, loose fluffs which partly sank to the bottom of the bouillon, whereas Bu grew rather similarly to B, except that the bouillon over the sediment was always rather turbid and fluffy.

Thus we now had to do with four variants, which grew differently on the most ordinary substrata and which retained their peculiarities if the resowing was only sufficiently frequent.

As we have already mentioned, V always divided itself on

being cultivated in bouillon into V and M, and on longer cultivation in agar stab in extract agar into V and B. M and Bu on the other hand remained quite unchanged, even after very long cultivation in both solid and liquid substrata, and never showed signs of going back to V and B. This division of V into M and B, when first it commences, rapidly proceeds, so that M and B quickly dominate the spreadings and finally V quite disappears. Once this happens it is not possible to get hold of it again. This latter phenomenon we have experienced ourselves with the museum culture we have worked with here.

It was not only in a morphological respect that these four variants differed. In regard to agglutination, it proved that the typical bacillus if we may so call it, V, agglutinated a Group III serum to the titer limit while none of the other variants showed the slightest trace of agglutination. (With agglutinations we always worked with suspensions of twenty-four-hour agar cultures in bouillon with a little formalin added as this proved to be the only manner in which we could obtain clear results.)

We then made agglutinating sera from rabbits for the variants V, B and M. As far as V and B are concerned we got excellent agglutinating sera, whereas M showed no power of agglutination. Table 1 shows the most important of the agglutination results, partly by direct agglutination and partly by agglutination after absorption. As this table shows, one may justifiably call these variants different types in a serological sense.

If we pass to the results found by fermentation tests with a number of sugars, differences are also apparent (see table). While V and M remained the same, it proved that B and Bu fermented a number of sugars which V and M never touch.

In view of results like these a number of theoretical questions arise, but we have decided to limit ourselves to a sober description of the actual circumstances as we have found them in this culture. Recent experience in this laboratory seems to show that these divisions also take place in the human organism, so that in a practical diagnostic sense too it is a problem of importance. For instance we have found strains of bacteria which altered the way in which they grew and fermented sugars like bacilli belong-

ing to Group III which only agglutinated with a B serum which is different from all the strains which we possessed in the laboratory.

In conclusion we will only go a little further into the question of the excessive autolysis mentioned above in the case of B which

TABLE 1

	1/25	1/50	1/100	1/250	1/500	1/1000	1/2500	1/5000	1/10,000
Group III Serum + V	+	+	+	+	+	+	(+)		
Group III Serum + M	0	0	0	0	0	0	0		
Group III Serum + B	0	0	0	0	0	0	0		
Serum V + V	+	+	+	+	+	+	+	0	0
Serum V + M	+	+	0	0	0	0	0	0	0
Serum V + B	0	0	0	0	0	0	0	0	0
Serum V + Bu	0	0	0	0	0	0	0	0	0
Serum V absorbed with V + V	0	0	0	0	0	0	0	0	0
Serum V absorbed with M + V	+	+	+	+	0	0	0	0	0
Serum V-B + V	+	+	+	+	+	+	(+)	0	0
Serum V-Bu + V	+	+	+	+	+	+	(+)	0	0
Serum M + M	+	+	(+)	0	0	0	0	0	0
Serum M + V	0	0	0	0	0	0	0	0	0
Serum M + B	0	0	0	0	0	0	0	0	0
Serum M + Bu	0	0	0	0	0	0	0	0	0
Serum M absorbed M + M	0	0	0	0	0	0	0	0	0
Serum M-V + M	+	(+)	0	0	0	0	0	0	0
Serum M-B + M	+	(+)	0	0	0	0	0	0	0
Serum B + B	0	0	(+)	+	+	(+)	(+)	0	0
Serum B + Bu	+	+	+	+	+	+	+	+	(+)
Serum B + V	(+)	0	0	0	0	0	0	0	0
Serum B + M	(+)	0	0	0	0	0	0	0	0
Serum B-Bu + Bu	0	0	0	0	0	0	0	0	0
Serum B-B + B	0	0	0	0	0	0	0		0
Serum B-M + B	+	+	+	+	+	+	(+)	0	
Serum B-M + Bu	+	+	+	+	+	+	+	+	0
Serum B-V + B	0	0	(+)	+	+	+	(+)		0

involuntarily led our thoughts to the bacteriophage of d'Herelle. In several respects, however, after a little closer investigation, it presented differences from this phenomenon. For instance it

is very seldom that one can spread from a colony infected with d'Herelle's "virus" and rediscover the "virus" in all the new colonies regardless of how vigorously the spreading is done. Spreading is indeed one of the methods of dividing the "virus" from the bacteria.

On filtering twenty-four-hour cultures of B through a Berkefeld filter we really succeeded in obtaining a filtrate which apparently had a lytic effect upon various bacteria, most pronounced for M and a Shiga-dysentery bacillus, so that a few drops added to 10 cc. bouillon simultaneously with the sowing of the bacteria in question, completely prevented growth, but in the tubes in which the concentration was less, the growth was only transitorily prevented. It was not possible to prove any increase of the lytic agent.

TABLE 2

	DULCITOL	MANNITOL	ARABINOSE	d-GALACTOSE	LEVULOSE	MANNOSE	LACTOSE	MALTOSE	SUCROSE	GLUCOSE	DEXTRIN	XYLOSE
V	−	+	+	+	+	+	−	−	−	+	−	−
M	−	+	+	+	+	+	−	−	−	+	−	−
B	−	+	+	+	+	+	+	+	+	+	+	+
Bu	−	+	+	+	+	+	+	+	+	+	+	+

On the agar surface it was also easy to show the very great restraining influence exerted by filtrates of quite young bouillon cultures. The experiment was repeated in this way: An area, 4 cm. square was inoculated with the bacilli in question, after which filtrate was added in varying dilutions. One drop of filtrate diluted 1:200 was able, when spread over the area named, to prevent completely the growth of both M and the Shiga-dysentery bacilli, while several other bacilli were apparently not restrained even by the concentrated agent.

It was very interesting to see that often, after incubation lasting two days or so, some few scattered colonies appeared on the area treated with the filtrate. These bacteria proved on

closer observation to be insensible to the filtrate, that is to say resistant, as we know it from bacteriophage researches.

Neither V, Bu, nor M formed any lysin which we were able to demonstrate by a similar method.

STUDIES IN MICROBIC HEREDITY[1]

I. OBSERVATIONS ON A PRIMITIVE FORM OF SEXUALITY (ZYGOSPORE FORMATION) IN THE COLON-TYPHOID GROUP

RALPH R. MELLON

Rochester, New York

Received for publication, February 4, 1925

INTRODUCTION

This series of studies is being written on the assumption that all thoughtful bacteriologists will welcome information that really comes to grip with the realities behind such problems as the following, viz., the reason for the inagglutinability of freshly isolated typhoid strains, the origin of spontaneous agglutinability in salt solutions, the origin of cultural and serologic heterogeneity as shown especially among the enteric organisms, the normal mechanism for the origin of variants and biotypes in pure line strains, the essential homogeneity of the three so-called species of organisms causing blastomycosis, and the significance of secondary or daughter colonies among the bacteria. The studies also involve an inquiry into the real significance of the pleomorphic cycle and a clue is afforded to that most interesting of medical questions, viz., the origin of virulence under natural conditions.

These studies aspire to interpret morphology, broadly speaking, in the *light of function;* to *associate*, but not to identify changes in function with certain *definite* morphologic alteration. Physiology has been made to serve always as the court of last resort when morphologic change appeared important. It has been nearly fifteen years since we were started on these studies by reason of certain observations, which current bacteriology

[1] From the Department of Laboratories, Highland Hospital.

could not then explain, and as we clearly see now, never can explain. In the search for a satisfactory explanation many similar observations were uncovered, now easily verifiable matters of fact, which demand explanation instead of prejudgment. *Hints*, in the form of studies previously published, have indicated all too inadequately the far reaching theoretical and practical importance of thoroughly controlled observations in the field of bacterial genetics.

In brief, the method of attack has been to insure pure line cultures by isolation of the single cell, hundreds of which have been segregated as occasion required. Furthermore, the technician of the modern laboratory has had no place in these studies; indeed the services of no second person has entered into any essential portion of the work, even to transplanting cultures

PREVIOUS WORK

From the earliest days of bacteriology until the present time occasional workers have observed what they have believed to be evidence of a sexual cycle for certain of the bacteria, but the view has never met with general acceptance. This has been the result, partly of the very scant and fragmentary character of the evidence, based as it was entirely on morphology and at best only suggestive of a series of changes that were to culminate in sexual reorganization. Even such a strong proponent of the idea as Löhnis (1921) admits that a great deal of what has been thus far reported upon the problem is rather unsatisfactory.

This, together with the fact that medical bacteriologists have regarded the fundamental biology of bacteria as a thing apart from their interest, has not permitted them to be as receptive toward work in this field as might otherwise have been the case. Contrast for example, the ready acceptance of Schaudinn's discovery of the *Spirochaeta pallida* as the cause of syphilis with the general apathy in regard to his work on autogamy with *B. bütchlii* (1902) and *B. sporonema* (1903). In the same category are the names of von Prowazek, Hueppe and others (see Löhnis). The fragments of work in this field are collated by Löhnis (1921) in his review of the life cycles of bacteria, which

makes it unnecessary for me to consider to any extent the literature of the subject. It is probably fair to state that nothing of a convincing nature has yet been accomplished.

It is our purpose to show that there is a process occurring with bacteria which appears to parallel in most of its details the process of zygospore formation which has been proven to occur among yeasts, and furthermore that the forms so produced may play a rôle of primary importance in the further evolution of the culture in its physiologic, biochemic and immunologic aspects. The essentials of this study were first presented before the Society of American Bacteriologists in December 1921, an abstract of which appeared in the Abstracts of Bacteriology (1922a).

EXPERIMENTAL

The strain of *B. coli* which we shall designate as Nx was isolated from the urine of a case of pyelitis in which it grew with such pleomorphism as to suggest a fungus. In addition to long filaments, some of which branched, many very large coccus-like forms were encountered developing from the filaments (fig. A). It was clearly shown that this remarkable pleomorphism was quite dependent on the fact that the patient had been taking utropin and sodium acid phosphate, to which she proved quite intolerant. Taken off the utropin the pleomorphism quickly disappeared, nothing but normal looking *B. coli* being found (fig. B). Fungoid developments would immediately reappear when she was given the utropin.

That there was nothing specific in a narrow sense about this environment was proven by the fact that other environments which could be better controlled also developed a similar picture, although on plain agar and broth the organism grew as does the usual coli organism. The conditions for zygospore formation will be discussed more in detail presently, but it will be pertinent here to outline the preparation of the media in which best results were obtained with this organism.

Broth was prepared in the usual way from veal, but in addition to the 0.5 per cent salt and 1 per cent Difco peptone, 1 per

cent of disodium glycerophosphate was added to the medium. It was autoclaved at 15 pounds pressure for from fifteen to twenty minutes and the resulting precipitate, which was moderate in amount and produced no change from the original pH of 6.8, was filtered off and the broth again autoclaved the same as before. A moderate amount of precipitate formed with a marked change in the color to a dark brown or mahogany. After several days in the ice-chest this precipitate entirely redissolved, leaving a perfectly clear broth with a pH of between 6.8 and 7.

A culture of the strain Nx, isolated from a single cell was seeded in the broth at 37° and was kept there for 72 hours, after which it was placed at room temperature (Baerthlein's experiment). Pellicle formation and sediment in addition to turbidity were present. Every day or two this culture was streaked on an Endo plate and incubated at 37° for from eighteen to twenty-four hours. The Endo agar had the same broth base as described above and enough of the dye was added so that the plate became colored after twenty-four hours in the incubator. This medium was unsuitable for coli-typhoid differentiation, due probably to its pH of 6.8. The Endo was prepared according to the directions given in Stitt's "Practical Bacteriology" except that the reaction was 6.8.

The colonies were of the spreading rough type and at the periphery were made up of rather long interlacing threads. It was among these threads, the so-called fungoid forms of the organism, that zygospore formation was observed to best advantage although it was also found among the normal forms, and under other conditions to which we shall refer. At room temperature the process tended to disappear, but reincubation resulted in further spreading of the colonies with a reappearance of the pleomorphic picture.

Figure 1, taken from the work of Guillermond and Tanner (1920) gives a diagramatic view of the relatively simple process of isoganic conjugation or zygospore formation, which is clearly paralleled by figures 2–5 inclusive of *B. coli*. These figures represent different stages of the process shown in figure 1, which consists essentially of a fusion of adjacent cells of a filament,

looking to the ultimate formation of a zygospore. Figure 1 shows that the process may occur indirectly by means of peduncles.

That this modification of the process also occurs with *B. coli* can be seen to good advantage in figures 6–10 inclusive. It should be noted, however, that this modification occurred in an eighteen-hour broth culture from a colony that appeared as a precipitate in the broth employed. This broth did not contain sodium glycerophosphate but was a veal infusion—1 per cent peptone and 0.5 per cent salt mixture which was brought to a pH of 7.4 with sodium hydrate, filtered and autoclaved. The fact that the strain was spontaneously agglutinating in this broth, whereas the original strain was not, indicates definitely that it was a variant although its agglutinative reactions with the anti-serum from the original strain were unchanged. The variant organism was derived as follows:

To a modified Endo plate, seeding was made from a modified glycerophosphate broth (as previously described), which had stood at room temperature for two months following three days incubation at 37°. In addition to colonies containing organisms of normal morphology were spreading rough ones with many pleomorphic fungoid forms. The latter spontaneously agglutinated in "normal" broth.

In figure 2 one observes a loop formation in the central thread-like figure, which is interpreted as an initial stage in the process. The loop is made up of two distinct cells which are part of the filament. In figure 3 the large goose-neck like organism shows two long, rather wavy chromatin filaments completely fused at the acute angle where they join, but as yet unfused in the distal portion. Note particularly in the right hand filament the spiral character of the chromatin, a most important feature as will be amplified later. The cell protoplasm surrounding this chromatin is not stained satisfactorily. Figure 4 shows a more mature zygospore. The stain was made intravitally. That its relation to the filaments in this picture is not artificial was proven by inducing currents in the specimen, thus causing these filaments to lash back and forward and the whole

specimen to change its position. The zygospore was not detached. Figure 5 shows another interesting form with unstained sporoid-like areas whose frequent association with these forms I have come to regard as an index of chromatic reorganization. It seems to spell an instability that often results in a new race under a suitable environment. Although no attempt has been made to study the fate of these spore-like bodies with this strain it is entirely conceivable that under a suitable environment they would rupture the cell and again germinate. We (1919) have shown this to be the case some years ago for another organism and the observation has been recently confirmed and extended by Tunnicliff and Jackson (1925).

Figure 6 shows an early stage of the process characterized by peduncle formation. In figure 7 the two coccoids arising from stalks either overlap or are incompletely fused. In these filaments indications of the spiral arrangement of the chromatin is imperfectly made out. In figure 8 indications of two stalks can be seen at the point of division between the organisms but no fusion line can be made out. Much the same is true for the large form of figure 9 where the spiral chromatin enters directly into the formation of the zygospore. In figure 10 we see apparently the end results of both modifications of the process occurring after twenty-four hours, and characterized by a very large number of giant coccus forms, some of which have a definitely thickened cell wall suggestive of the yeasts. Note, too, the intracellular differentiation in the chromatin.

In the filament of figure 11 is seen the tilting of the chromatin at the fusion point between two cells. This is one of the earliest stages in the process which is more advanced in figure 12. This is what I call the double golf club form on account of its fancied resemblance to two clubs with their heads in apposition. Note that no fusion has occurred.

Figure 13 shows three of these forms in one field and in different stages of development. The figure to the left at *a*) shows an indistinct line of separation between the two coccoid portions which have not yet completely fused. The figure to the right at *b*) shows two stalks present but fusion is complete, while the

figure at *c*) shows the very faintest line of fusion of the two unequal segments. A separate peduncle can be made out in the negative for the smaller segment. In figure 14 no evidence of a fusion line is present.

In our development of this subject we have made it a point to study this process as it occurs in the large fungoid phase of the cultures, and to find an environment that would help evoke this phase. The interpretation of the picture is made more certain in these larger forms where the details of the process are much more clearly delineated. The implication that the cultures may possibly be mixed was forestalled by starting them from single cells. This was made especially necessary owing to the enormous size that some of the forms developed. Figures 15 and 30 for example grew in a medium prepared as follows:

The patient's urine from which the strain was isolated was buffered to a pH of 4.8 with KH_2PO_4, the patient being on utropin at the time. The resulting precipitate was filtered off and the filtrate passed through a Berkfeld filter. Since the organism refused to grow in the urine so treated, about four drops of sterile human blood serum were added to 2 cc. of it. In twenty-four hours at 37° the organism grew moderately well, but produced many large pleomorphic forms, which were further magnified by intravital staining and photographic reproduction at 3500 diameters. This enabled the resolution of surprising detail as seen in figure 15 and figure 30. A consideration of many similar forms showing changes in the reproductive mechanism of the cell is left for a future communication.

Figure 15 is deserving of attention aside from its size. If seen among the protozoa or other higher forms, one could consider that the micronuclei of two organisms were in contact, which is regarded as the *sine qua non* of true conjugation among certain of the paramecia. One could even consider the forms as differentiated into micro- and macrogametes. Without more study the form is of suggestive value only; yet figure 30 from the same culture shows a nuclear reconstitution that takes the form of a tangled skein; furthermore, it is easy to get the impression of a doubly contoured membrane. Such a structure

suggests an encystment, a change common to higher forms, which *usually follows conjugation* or the closely allied process known as association of nuclei.

The significance of the large fungoid and branching forms in bacterial cultures is unfortunately not as well recognized as it should be, since bacteriologists have ceased to be interested in this phase of the subject. Although zygospore formation can be seen to better advantage among the larger forms, it occurs just as certainly among the normal sized forms of the organism as indicated by the series of figures shown from 16 to 22 inclusive. Where several organisms are present in any one field the arrow points to the ones of significance.

In figure 21 an infrequent but interesting modification of the process occurs, as is shown by the long sausage-like filament at its left end. Here a moderate sized circular body is attached to two cells by very slender filaments. An earlier stage of the process seems to be shown by figure 22 where the converging filaments have not yet developed a circular body at their junction. Although figures 16 to 22 are from an eighteen-hour broth culture this process, among the more normal sized forms of the organism, also occurs on an agar plate as evidenced by figures 23* to 26 inclusive. These forms were especially numerous in the secondary colonies developing on an Endo plate which had been incubated for twenty-four hours at 37°, placed at room temperature for a few days and then in the ice-chest for a week, where secondary or daughter colonies arose from the flat spreading colony. Although most of our figures are taken from relatively young cultures the process may continue in older cultures under somewhat different conditions—in this case at lower temperature.

The double golf club form of figure 26 is comparable with figures 12 and 27, but the filaments of the latter delineate the process more clearly than the forms of normal size in figure 26. However, figure 27 is only a more highly magnified picture in an unfixed and hence unshrunken specimen, while figure 26 had been fixed.

Note also in the filaments of figure 27 how the wave-like core

*Fig. 23 should be reversed.

of chromatin is differentiated from the cell plasma, and how by direct extension it contributes to the formation of the developing zygospore. Figure 28 shows a similar form but fusion is more complete as indicated by the slight indentation at the top of the zygospore.

ZYGOSPORE FORMATION IN RELATION TO UNINTERPRETED PLEOMORPHIC ANOMALIES

a. The giant whips of Loeffler and Novy

Many years ago Novy (1899) described certain curious structures found chiefly in the condensation water of motile bacteria which he termed giant whips. They occurred chiefly in two forms: *First*, as a single long slender spiral-like structure taking origin from the end of the bacteria, and *second* as a more complicated braided form not unlike the strands of a rope, usually spindle shaped. They were not flagella since they stained with simple dyes, although he was inclined to relate them to the flagellar mechanism of the cell. They were not motile.

Perhaps the most constant concomitant feature of zygospore formation is the *spiral reorganization of the chromatin in the filaments*, to which we have already alluded. This interesting structure often enters directly into the formation of the zygospore (figure 3, 6, 7, 9, 27, 31). It may appear as a slender spirochaete-like central core running through the filament, or it may have a very distinct roped appearance as seen in figures 31b and 32. The zygospore is seen forming at *a* in figure 31 and the spiral arrangement is present in the filament to its left. Indications of the manner in which this rope-like structure is differentiated are seen at *b*, while in figure 32 the strands are fully and strikingly differentiated. In figure 29 the simpler, less undulating type of spiral emerges from the end of the bacillus. These structures stained with the simple dyes and were nonmotile.

In figure 30 the skein-like character of the chromatin is particularly well seen and its probable sexual significance has already been discussed. It is well known that among many of the pro-

tozoa this chromatic reorganization known as the "Wurst formigeschlingen" *is the most constant mark of sexual reorganization.* The confirmation of this fact by observing what is regarded as a similar process among the bacteria is significant, and it seems entirely possible that Novy's giant whips and our spiral chromatic reorganizations are essentially the same. Certainly their characteristics and the mode of their origin as suggested by these figures, explains their arrangement better than the generally accepted view proffered by Loeffler, viz., that they were woven masses of ordinary whips.

b. Meyer's bacterial plasmodia

Rather closely related to these structures is what A. Meyer (1912) spoke of as a plasmodium which occurs normally among certain fungi. It consists of a thread-like connection between two cells. It may be short or very long as seen in figure 29. In figures 33–37 inclusive, we see the short form of this thread-like connection between cells which at the middle point may swell out into a very definite chromatin granule. This is seen to advantage in figure 34, and a hint as to its significance is shown in figure 35 where a true branch is seen definitely arising from this granule. In figure 36 the resemblance to the zygospores as they occur among the normal forms of the organisms, e.g., in figures 11 and 12, is noteworthy. In figure 37 at *a* is a similar form somewhat further developed; it is seen to be associated with a very definite zygospore as shown at *b* of figure 37. The beak-like structure seen at *c* was regarded by Hueppe many years ago as an index of sexual reorganization. I might add that in the urine from which this culture was originally isolated this slender intercellular thread-like structure occurred in such abundance as to form a matted network. It was easy to trace its origin from the end of the cell, or as a "naked" chromatin filament connecting two cells (fig. 29). It frequently branched.

c. Arthrospore formation

The arthrospore, which in current bacteriology is practically obsolete, was originally regarded by Hueppe as a large coccoid

form of special resistance occurring among the cocci. There is no question that such forms among both bacteria and cocci do have a special resistance although not to heat. As I shall show, however, in a subsequent study I believe they have the same significance as they do among the bacillary forms, namely, a form of reorganization which makes possible the evolution of new developmental stages, or variants, if you please. Physiologically speaking they can be regarded in the same light as the zygospores of the bacilli.

Several years ago I (1920) published a study which showed clearly that these large forms were the immediate precursors of a new line of true cocci. As is shown in figures 38 and 39 their origin in this case from the so-called *B. Hodgkini* appears to be similar at times to that among the enteric organisms.[2] We designated them then as giant coccoids, their true biologic

[2] The mechanism for the formation of these forms in the diphtheria group has not been worked out in detail as has been the case for *B. coli*. That they are always formed by the same mechanism seems doubtful. The fact that they are resting forms of increased resistance, as well as the seat of mutation change is of paramount importance physiologically, regardless of their morphologic origin or the name that we attach to them. Their physiologic similarity with the forms whose origin is clearly that of the zygospore seems to justify my presumption in regard to most of these large coccoid forms that I know, viz., that fundamentally they have in common the reorganization necessary for the preservation of the life of the species, but in the extent of this reorganization they may differ among themselves. This difference has found expression in a host of names such as chlamydospore, arthrospore, "daurnenzellen," gonidium, gonidiangium, acladial formation, zygospore, in addition to the meaningless term coccoid and the frankly erroneous term *involution form*.

If one but keeps in mind that, broadly speaking, they may be viewed as branches of the same "reorganization tree" as is attested to by their physiologic similarity, one will not attach a too hard and fast significance to nomenclature. However, in the concrete case when the mechanism of their formation and their physiology is not clear, I like to regard them tentatively as gonidia, while still appreciating the progressive modifications that even these may undergo under suitable conditions.

For example, among the yeasts it is common for copulation to precede asc formation, in which case the zygospore becomes the ascospore. They are stages of one process; And among the bacteria, as we have shown (1919) and as Tunnicliff and Jackson (loc. cit.) have recently confirmed, the gonidium may become the gonidiangium which differentiates intracellular individuals capable of germination. Indeed one might regard their gonidiangium as the homologue of the asc.

nature being unknown to us. In figure 40 the differentiation of these forms, as described in the paper referred to, can be seen. Tetrad formation is very prominent and although this culture has settled down into a straight diplococcus it frequently shows very large coccoid reversionary forms. In a culture of *B. alkaligenes* isolated from the body in this large diplococcoid form we have been able to effect its development into a very long bacillary or thread form, thereby confirming the real significance of these forms among the cocci (1925).

A recently published study (1922) gives further evidence of the physiologic significance of zygospores in respect of the development of new races of organisms. In this instance I have shown that the descendants of these coccoid forms (zygospores?) change permanently their stability in salt solution, but their germinating temperature was quite different from that of the antecedent bacillus which gave rise to them. The new stable race was serologically identical with the spontaneously agglutinable antecedent bacillus (N. D. strain).

In the Nx strain (*B. coli*) we are able to *associate* definitely changes in fermentation of lactose with the development of these coccoids. The new strains showed great delay in acid formation while gas production was lost. Furthermore, we have been able to associate definitely changes of agglutinability of *B. typhosus* with this process. That it may occur in *B. typhosus* is clearly seen in figures 41 and 42. At this stage we can isolate a closely related biotype which is much less agglutinable than the antecedent type. The new type may take origin from the zygospores as was the case when a change in stability occurred with the N. D. strain. A derived variant of this same strain of typhoid bacillus shows development of the process in the large fungoid phase of the culture as previously shown for the Nx strain of *B. coli*. Various stages of this development are reproduced in the filamentous forms of figures 43 to 48 inclusive. This strain is quite inagglutinable.

Although the details of this quoted work will be forthcoming in subsequent communications it seems desirable to anticipate thus briefly, so that the skeptical may appreciate that our evi-

dence for thus "vitalizing" *his involution forms* is based on something beside pure morphology. *It is based on a process which throughout the realm of biology is known to condition, almost, the development of new races and new characters.* Darwin (1867) appreciated that "beings produced sexually are much more liable to vary than those produced asexually," although he did not foresee what later experimental biology has indicated, namely, that evolution itself appears to have the process of mutation as its mechanism. This correlation of variability and sexuality is not intended to imply that organisms conjugate, *simply that they may vary.* Primarily, of course, sexual reorganization is a means of rejuvenation of the physiologic activities of the organism to secure the indefinite life of the species. It is by no means necessary that variation result, although such potentiality is brought nearer the surface by the disturbance of equilibria that sexuality itself implies.

In connection with our studies on *B. typhosus* and the N. D. diphtheroid strain just referred to it is pertinent to quote the work of Almquist (1924), whose claims for the sexuality of bacteria rests in part on the hybridization of *B. typhosus* and *B. dysenteriae* by growing them in mixed cultures. The criterion for the recognition of the hybrid is the agglutination test: the hybrid agglutinates in both sera whereas the maternal strains are specific. In light of the relative ease with which it is possible to derive variants of *B. typhosus* of either increased or decreased flocculability it would seem essential to show that this hybrid actually contained agglutinogen for both *B. typhosus* and *B. dysenteriae.* The experiments would have been more convincing had this been done, of which there is no evidence.

Convincing demonstration of such a spectacular feat is beset with another difficulty evidenced by the work of Meyer and Neilson (1920), who have shown that pure cultures of the single species *B. typhosus,* may acquire specific agglutinability for an antienteriditis serum as well as adsorptive capacity for the enteriditis agglutinins of the serum. This makes it necessary in hybridization experiments to rule out "spontaneous" variation or the purely symbiotic influence of the accompanying strain.

Almquist (loc. cit.) also indicates the "haploid" and "diploid" arrangement of the nuclear chromatin as a mark of sexuality. I have frequently observed this with the diphtheroids especially with my N. D. strain when the reorganization associated with the diploid arrangement is likewise associated with the development of a distinct growth stage or variant.

The great adaptability (variability) of microörganisms may permit a strain to lose its sexual and gonidial phases under conditions where previously they occurred in abundance. For example: The *B. Hodgkini* of figures 38 and 39 grew sparsely in the small bacillary form for many years after isolation, maintaining their viability as well as their ability to grow at 37° by forming these large coccoid forms, which were much more durable than the bacillus. In time the growth of the latter gradually became more luxuriant, pigment formation slowly appeared, until it is now viable over long periods of time without the formation of this special coccoid stage.

Serologically it is clear that this organism is a *closely related variant* which dissociated spontaneously under conditions where variation so frequently occurs, namely, in old cultures, the original form of which is no longer viable. Here again is closely revealed the important consideration that an *adapted* strain is a *variant* strain, and is not *primarily* the result of an increased metabolism on the part of the organism. Although in this instance no mechanism of dissociation was traced, the fact that the culture was a pure line one leaves no doubt of the correctness of the conclusion and exemplifies the decisive value for genetic purposes of working with single celled strains over a course of years. Especially is this true when one has taken the precaution of developing immune sera for the original pure line strain.

The inadequacy of current bacteriologic theory to recognize the precise nature of such adaptability has undoubtedly resulted in confusion where attempts have been made to reproduce variation experiments with the *supposedly* original strain. Important genetic changes may occur in a strain which are accompanied by little or no serologic modification. When the change

in the strain itself results in a race in which this coccoid or gonidial stage in its life history is suppressed, it is apt to be unfit for variation work, short of formulating a new set of environmental conditions that will evoke this important stage in its life cycle.

CONDITIONS UNDER WHICH ZYGOSPORE FORMATION DEVELOPES

Accordingly it is scarcely to be expected that one should be able to formulate a fixed set of conditions in which this process should occur, even with a single group of organisms. It is possible, however, to lay down some general principles which often are of great help in its development. Zygospores have usually been observed to occur under conditions of marked aerobiosis such as pellicle formation and on agar slants. It is most clearly seen in the large filamentous phase shown in many of the photographs. This may occur naturally or special conditions may evoke it. Walker and Murray (1904) have observed that certain of the anilin dyes produce filaments, although they leave the interpretation an open question. An acid reaction as we have indicated is often of advantage. The process usually occurs within eighteen or twenty-four hours at 37°, although as has been indicated spores also develop in the secondary colonies of older cultures at icebox temperature.

The old experiment of Baerthlein has helped to produce them. Baerthlein's method of producing variants was to grow an organism in broth culture for several days or longer at incubator temperature and to plate from this ageing culture at intervals. This method of producing variants has been successfully repeated by many workers, and as I have indicated it was of value with our Nx strain in a modified broth, especially when the plating was made to modified agar, further changed by the Endo feature. The philosophy of the Baerthlein experiment has never been understood but the sum total of our experience indicates that very often the reorganizations so developing are along the lines here laid down, which would explain its success in developing variants. Lytic action for the young forms of the cultures has been frequently observed, and the recent experi-

ments of Arkwright (1924) suggest the possible relation of such lysis to transmissible lysis or the bacteriophage. The rôle of the bacteriophage in producing the large coccoid forms which yield variants has been shown repeatedly by d'Herelle (1922) and others.

Although in the years that we have studied this process we have observed literally thousands of these forms in different stages of development, we have attempted as yet but little warm stage work. The *à priori* considerations that seem perhaps to make such approach desirable fail to consider that the germination of these forms often demands a changed and somewhat selective environment which usually transcends the limitations of the warm stage method. Even when positive the method would not be conclusive as a criterion of variability, for such multiplication might be considered by the skeptical as abortive in character. Inasmuch as our primary interest lay in a mechanism for variability the impractical character of this approach should be clear.

On the other hand, leaving out the question of variability the warm stage method has shown that certain pleomorphic forms do reproduce freely, if atypically. Accordingly those who strongly prefer the method may find solace in the work of Hort (1917) with *B. typhosus*, of Bergstrand (1918) with *B. diphtheriae* and our own study of a fuso-spirillary organism (1919). Furthermore, a very recent study by Gardner (1925) from Dreyer's laboratory in the University of Oxford, answers the often raised question in respect of the capacity for reproduction of the large branching (involution?) forms of the enteric group. By direct examination under the microscope he has clearly shown for seven species of this group that the branching "Y" forms may yield normal bacilli from any or all of the three points of the "Y," which phenomenon he refers to as "Three-point Multiplication." Furthermore, he finds in certain cases that the "Y" forms are closely connected with recognizable variation within the species.

The physiology that we have shown, and will show, for the zygospore is just what one would expect on the pure assumption

that they are sexual in nature, and our primary interest still lies in the application of the idea to medical problems as indicated, rather than along pure botanical lines. Even so it is desirable that direct observation confirm the presumptive character of the evidence adduced. That the morphology of the process is so strikingly consonant with the sexual interpretation of its nature greatly strengthens the probability of its soundness.

CONCLUSIONS

1. It has been shown among members of the colon-typhoid and diphtheria groups that all stages of a process can be traced, which parallels in its morphologic details what is known as isoganic conjugation or zygospore formation, such as has been proven in recent years to occur with many of the yeasts.

2. Previous studies, as well as work to be presented in the near future, indicate clearly that these zygospores—erroneously known as involution forms—will germinate under the proper conditions.

3. Their germination and its direction as well, depends primarily on the extent to which they have been differentiated by the action of certain saturating environmental influences of which bacteriolysis appears to be but one.

4. When the new environmental conditions are suitable for germination of these zygospores, their descendants may be seen to have acquired new characters, or perhaps to have had latent ones rejuvenated. The fact that the acquisition of virulence has been associated with the cyclic development of such new races suggests a most important bearing on many questions of infection and immunity.

5. Under certain conditions then, this process may become a mechanism for the dissociation of the pleomorphic cycle as it naturally occurs in the life history of all organisms, and forces us to regard bacterial pleomorphism in the same way in which we regard it among the fungi. Thus is logically explained the occurrence of stabilized coccus and bacillary stages of microorganisms which may or may not be serologically related. Varia-

tion within pure lines of bacteria is thus, for the first time, given botanical correlation through the medium of a special evolutionary stage in their life history.

SUPPLEMENTARY NOTE

Some months ago, after the manuscript of this paper went to press, a recently appearing monograph entitled "Bakterien-Cyclogenie" was kindly loaned to me by Dr. Howard Brown of the Pathological Department of Johns Hopkins University. The author, Dr. Günther Enderlein (1925) of the University of Berlin, describes a sexual cycle for *V. cholerae* attended with actual differentiation into male and female gametes, which he speaks of as the spermit ♂ and the oit ♀. The cycle has been traced with the living forms.

In addition to a morphologic differentiation into two gametes that closely resemble the spermatozoon and ovum of the higher forms he observes that the spermit ♂ is endowed with an active motility, quite teleologic. As a result the contacts that the spermit makes with the oit effects its fertilization. These sexual cells are derived in an as yet unexplained manner from a coccoid structure, the gonit, which in turn has *its* origin from the well known gonidium. The latter change, which appears to be a reorganization of the chromatin along rather definite lines, he claims to have successfully traced.

It is of considerable interest that the gonidium after its transformation to the gonit is no longer viable on solid media, and even in peptone water, division figures in 5 to 7 hour cultures are notably few. Yet transplants from this broth to solid media result in luxuriant growth of *V. cholerae* with colonies much larger than the original strain. This paradox finds ready explanation in the circumstance that the peptone water, in addition to furthering the differentiation of gonidian into gonites, facilitates the purposeful migration of the spermit ♂ for fertilization of the oit ♀. The strain thus rejuvenated grows with unaccustomed luxuriance even on solid media.

This is one of the few instances where he shows the influence of the sexual process on the further evolution of the culture.

Although little more than a suggestion of the far reaching evolutionary changes that sexuality may condition in a culture, this instance is quite confirmatory of the central theorem developed in our published papers beginning in 1917 (see bibliography). This work, which has entirely escaped the attention of Enderlein, shows that the apparent non-viability *assumed* by current bacteriological theory for these "involution forms" was the result of reorganization changes within them which, when completed under a suitable environment, resulted in viability that was often associated with the appearance of new characters. Although his is a more complex process than the simple zygospore formation that I describe its end results are *physiologically* similar as far as his work goes. He deals with the field of mutation, chiefly in a speculative way.

It is of interest that with *B. typhosus* and *B. diphtheria* and many other species he has never been able to observe the differentiation of spermits and oits from the rather easily produced gonit stage, even though a variety of media was tried. Although it may be, as he suggests, that the proper conditions for their differentiation have not been met, in light of our own work with these two groups it seems more probable that zygospore formation or some other modification of the sexual process obtains with them. The apparent haploid-diploid arrangement of Almquist (loc. cit.) with our N. D. strain and the autogamy described by A. Williams (1910) for the Klebs-Loeffler bacillus are examples.

REFERENCES

ALMQUIST, ERNST: Investigations on bacterial hybrids. Jour. Inf. Dis., **35**, 1924, 341–346.

ARKWRIGHT, J. A.: The source and characteristics of certain cultures sensitive to bacteriophage. Brit. Jour. Exper. Path. **5**, no. 1, February, 1924, 23–33.

BERGSTRAND, HILDING: Uber den Klebs-Löfflerschen Bazillus. Acta Oto-Laryngologica. **1**, Fasc. 1, 1918, 131–186.

DARWIN, CHARLES: The variation of animals and plants under domestication. A. Gray, New York, vol. 2, viii, 1867, 568.

D'HERELLE, F.: The bacteriophage, its rôle in immunity. Williams & Wilkins, Baltimore, 1922.

ENDERLEIN, GÜNTHER: Bakterien-Cyclogenie Prolegomena zu untersuchung über Bau, geschlechtlich und ungeschlechtlich Fortpflanzung und Entwicklung der Bakterien. Walter de Gruyter & Co., Berlin and Leipsig, 1925.

GARDNER, A. D.: The growth of branching forms of bacilli ("three-point multiplication"). Jour. Path. & Bact., **28**, no. 2, April, 1925, 189–194.

GUILLIERMOND AND TANNER: The yeasts. John Wiley & Sons, New York and London, 1920.

HORT, EDWARD C.: The life history of bacteria. Brit. Med. Jour., May 5, 1917, 571–575.

LÖHNIS, F.: Memoirs of the Natural Academy of Sciences, vol. xvi, second memoir. Studies upon the life cycles of the bacteria. Part 1. Washington, 1921.

MELLON, RALPH R.: Study of the diphtheroid group of organisms with special reference to their relation to the streptococci. Jour. Bact., **11**, no. 2, **3**, **4**, 1917.

MELLON, RALPH R.: Contribution to the bacteriology of a fuso-spirillary organism, with special reference to its life history. Jour. Bact., **4**, no. 5, September, 1919.

MELLON, RALPH R.: The life cycle changes of the so-called *B. Hodgkini*, and their relation to mutation changes in this species. Jour. Med. Res., **42**, no. 1, 61–76, July–September, 1920.

MELLON, RALPH R.: (a) The diplococcus and bacillary stages of *B. coli* and their possible relation to growth cycles and sexuality. Abst. Bact., **6**, 1922, 24 (Abst. no. 59).

MELLON, RALPH R.: (b) Spontaneous agglutinability of bacteria in relation to variability and to the action of equilibrated solutions of electrolytes. Jour. Med. Res., **43**, no. 3, June–July, 1922, 345–367.

MELLON, RALPH R.: Biologic origin of cultural and serologic heterogeneity with pure line cultures of enteric origin. Abst. Bact., 1925 (in press).

MEYER, A. 1912. *Die Zelle der Bakterien*. Quoted from Bergstrand, "Über sogenannte Corynebakterien und ihre Verwandten nebst Bemerkungen über Bakterien im Allgemeinen," Acta Medica Scandinavica, **52**, no. 3, October 25, 1919, 1–94.

MEYER, K. F., AND NEILSON, N. M.: Irregular typhoid strains and the infections caused by them. Jour. Inf. Dis., **27**, 1920, 46–71.

NOVY, F. G.: Laboratory work in bacteriology. Geo. Wahr & Co., Ann Arbor, Mich., 1899.

SCHAUDINN, FR.: Beiträge zur Kenntnis der Bakterien und verwandter Organismen. I. Bacillus Bütschlii n. sp. Arch. f. Protisenk., **1**, no. 2, 1902, 306–343.

SCHAUDINN, FR.: Beiträge zur Kenntnis der Bakterien, etc. II. Bacillus sporonema n. sp. Arch. f. Protistenk., **2**, no. 3, 1903, 416–444.

TUNNICLIFF, RUTH, AND JACKSON, LEILA: *B. gonidiaformans* (N. SP.). Jour. Inf. Dis., **36**, no. 5, May, 1925, 430.

WALKER AND MURRAY: Effect of certain dyes upon cultural characters of *B. typhosus* and some other microorganisms. Brit. Med. Jour., no. 2270, **16**, July 2, 1904.

WILLIAMS, ANNA: Park and Williams: Pathogenic Microorganisms, pp. 17–18. Lea & Febiger, Philadelphia.

MAGNIFICATIONS AND PREPARATION OF SPECIMENS

The following magnifications correspond to the numbers of the photographs, which were made from specimens stained in their own liquor when possible. From solid media they were put in broth or NaCl solution 0.85 per cent, and stained with an aqueous solution of the dye bringing out the best detail—usually carbol thionin. Fixation artifacts are thus avoided while the organism appears somewhat larger than when fixed.

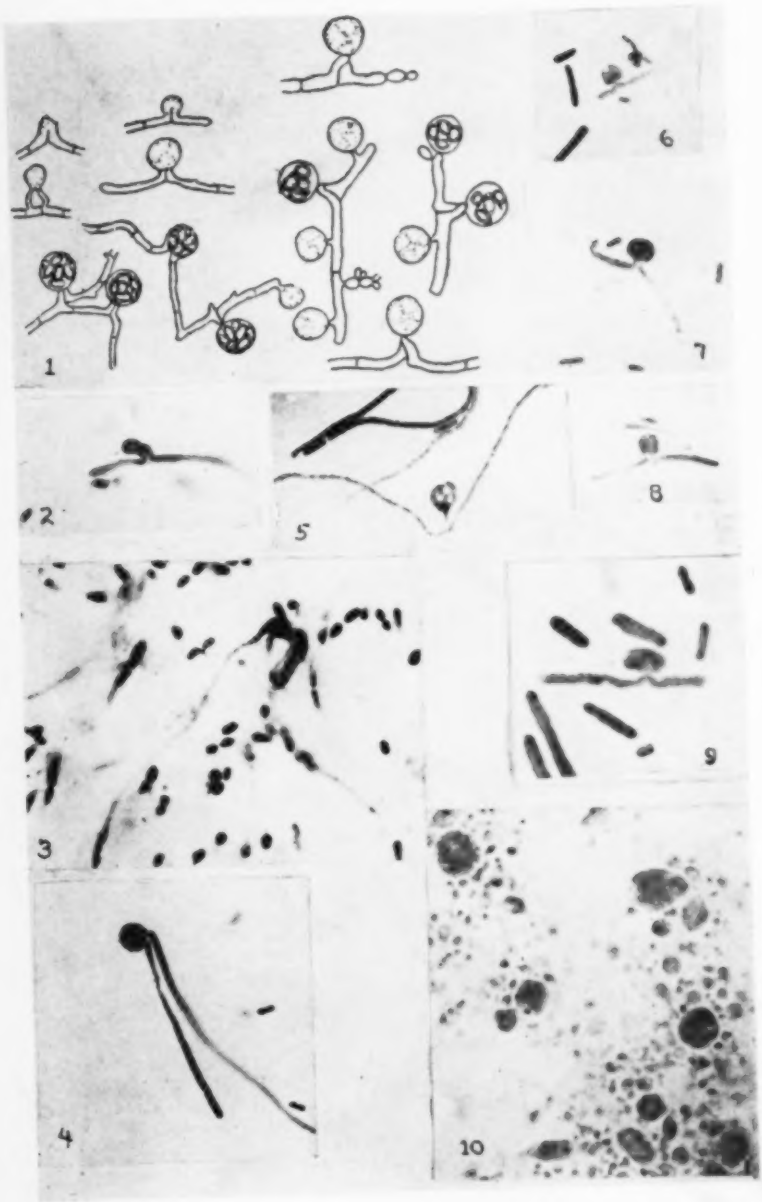
X-1400: Nos. 2–8 inclusive; 11–14 inclusive; 16–20 inclusive; 23–33 inclusive; 34–39 inclusive; 41–48 inclusive; Figure B.

X-1500: Nos. 27, 40, figure A.

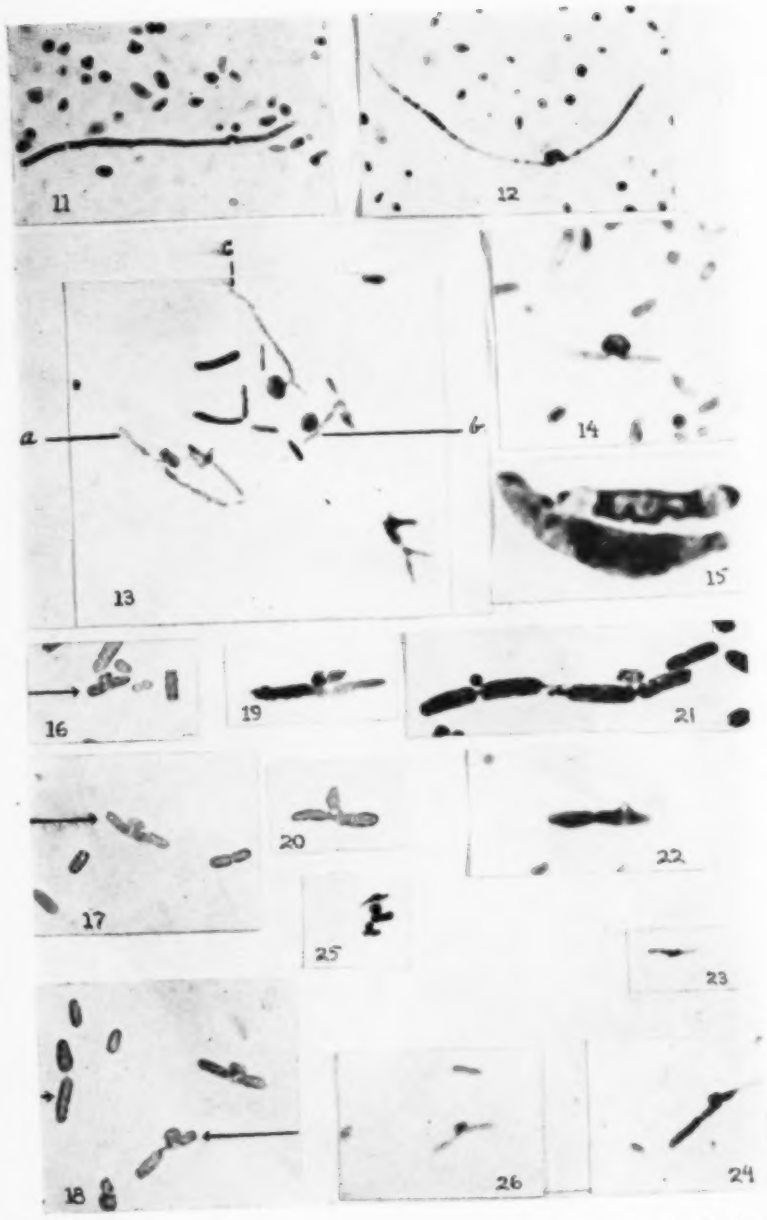
X-1900: Nos. 21, 22, 26, 28, 29, 32.

X-2000: Nos. 9, 10.

X-3000: Nos. 15, 30.

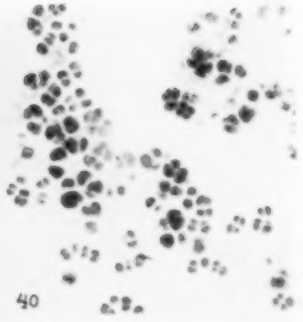


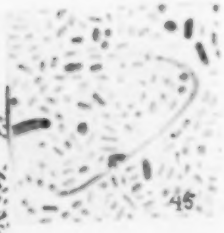
(Mellon: Studies in microbic heredity)

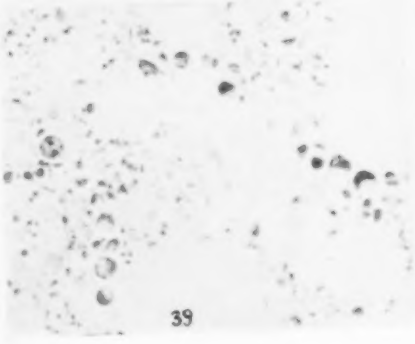


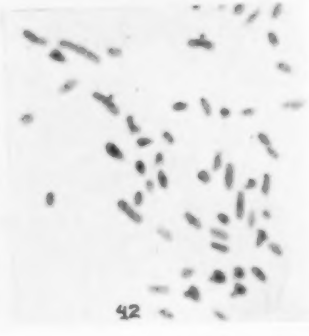
(Mellon: Studies in microbic heredity)

(Mellon: Studies in microbic heredity)

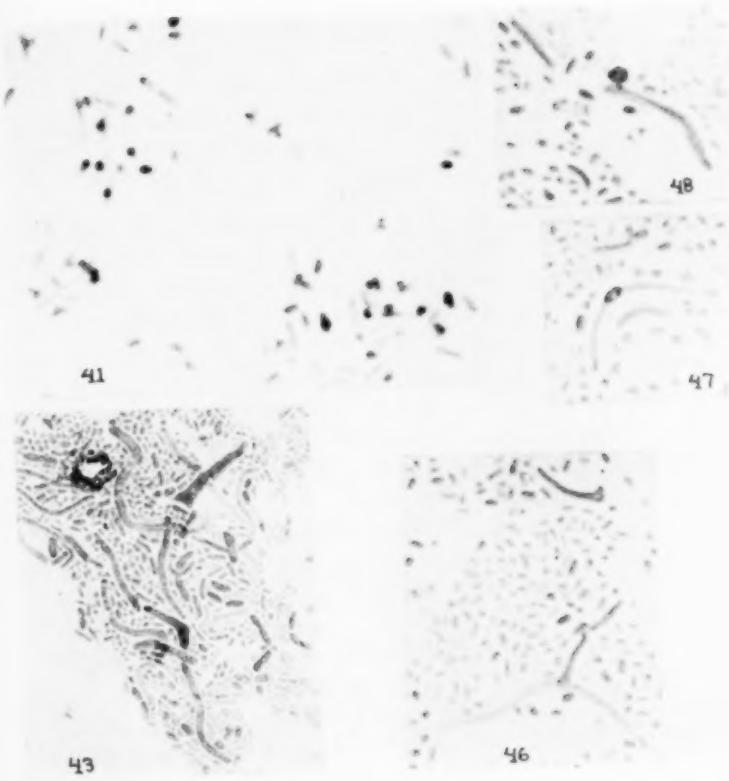








(Mellon: Studies in microbic heredity)


41
48
47
43
46

A STUDY OF ANAEROBIC BACTERIA

J. HOWARD BROWN

From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey

Received for publication November 11, 1924

The very excellent work done in the study of anaerobic bacteria isolated from wounds during the World War has indicated certain directions along which the sporulating anaerobes in general may be profitably studied. Some very useful media have been introduced, notably the cooked meat or cooked brain media. Better and simpler methods of securing anaerobic conditions have been devised, such as various developments of the anaerobe jar of McIntosh and Fildes (1916) and the use of vaseline on the surface of media in test tubes. The contributions of Wolf and Harris (1917, 1918, 1919), Bushnell (1922), Kendall, Day and Walker (1922), and DeBord (1923) demonstrated the usefulness of biochemical methods for the study of the anaerobes and the impossibility of understanding their activities without resort to such methods. The cultural and systematic studies of Weinberg and Sequin (1918), Reports of the British Medical Research Committee (1917, 1919), Robertson (1916), McIntosh (1917), Henry (1917), Adamson (1919), Heller (1920, 1921, 1922), and Hall (1922) have done much to untangle the descriptive literature of the past and to establish the identity of certain species and certain natural groups of anaerobes. With this background of methods and the beginning of a natural classification it was thought worth while to study a fairly large group of anaerobes taking particular care not to neglect the non-pathogenic species which from a biological standpoint may be very important members of the group. Work on the anaerobes has often been limited by technical difficulties, the use of large quantities of media, elaborate apparatus, difficult sampling, danger of con-

tamination, and lack of time and assistance. Simple test tube methods are much to be preferred and it is highly desirable that work on such a large and important group of organisms shall be brought within the facilities of as many workers as possible. The work to be reported has been largely influenced by these considerations.

MATERIAL

The material for the study consisted in strains from a number of sources. There were several strains from the collection of Dr. Theobald Smith, a strain of *C. putrificum* furnished by Prof. L. F. Rettger, a strain of *C. chauvoei* isolated from some "blackleg" muscle received from A. E. Hagan of the Kansas Blackleg Serum Company of Amarillo, Texas, a strain of *C. chauvoei* isolated from dried muscle and one of *C. vibrion-septique* from similar material sent by Dr. W. A. Hagan of Cornell University, another strain of *C. vibrion-septique* isolated from some infected guinea pig muscle supplied by S. H. McNutt of Iowa State College, and a strain of *C. tetani* isolated at autopsy from the castration wound of a pig with clinical tetanus. Strain C. 3413 was from the feces of a cow, strains C. 710Fa, C. 710Fb, and C. 710Fe from the feces of another cow, and strain Clf. 993 from the pleural abscess of a calf. In addition to the above there were isolated as many different anaerobes as could be found in the normal intestinal tract of a calf slaughtered when one month old. These strains came from the contents of a loop of duodenum, a loop of ileum, some of the contents of the cecum, and some feces from the rectum. For the present report the source of these strains is regarded as unimportant. What was wanted was simply a representative collection of anaerobes from some source in nature. From the intestine of the calf there were studied three strains from the duodenum, three from the ileum, eleven from the cecum, and six from the rectum. If possible duplicates from each source are excluded; there were at least two distinct species from the duodenum, three from the ileum, seven from the cecum, and six from the rectum. From the entire intestinal tract of this animal

there were isolated not less than fourteen different species. Most of those found in the cecum were present also in the rectum.

METHODS

The general plan of the study was as follows:

Thick suspensions of intestinal contents or fecal material were heated for fifteen minutes at 80°C. and then a drop or two of the suspension was inoculated into vaseline tubes of fermented veal infusion bouillon, fermented bouillon plus 1 per cent of lactose, and unfermented bouillon plus 1 per cent of glucose. Each tube of medium contained a small piece of rabbit or guinea pig kidney placed in the tubes before they were autoclaved. After incubation for three days each of the cultures was heated for fifteen minutes at 80°C. By this method sporulating aerobes were eliminated (Brown, 1922). The cultures were then plated in horse blood agar in petri dishes with clay tops glazed on the outside only and were incubated in the anaerobe jar (Brown, 1921). From the blood agar plates both deep and surface colonies were studied and as many different kinds as could be found were transplanted into veal infusion bouillon plus kidney tissue under vaseline. These cultures were studied and again plated on blood agar, the process being repeated until pure cultures were assured. No difficulty was experienced in obtaining and maintaining pure cultures and it is doubted whether the method of successive plating, careful study of the colonies and fishing from deep colonies is any less dependable than that of picking out single cells or spores with a micro-pipette.

The strains isolated were studied in cooked meat medium under vaseline with reference to change in color of the meat, consistency, digestion, and formation of gas. The formation of a black ring of deposit at the surface of the medium was taken to indicate hydrogen sulfide formation. Cultures in cooked meat were the ones employed for study of the morphology of the organisms.

The growth of the colonies in deep agar shake cultures afforded means for studying the shape of deep colonies and gave a rough index of the relative degree of anaerobiosis favorable for the

growth of each strain. In such tubes of agar the colonies grew to within from 3 to 15 mm. of the surface, the growth stopping abruptly at this level. Agar shake cultures also serve well as stock cultures and if a little sterile vaseline is pipetted onto the surface of the agar the medium keeps indefinitely, without drying and without contamination by molds.

Tubes of veal infusion gelatin under vaseline were inoculated in fluid condition and incubated with sterile control tubes at 35° to 37°C. for several weeks. From time to time they were taken from the incubator and refrigerated or placed in cold water. The control tubes never failed to solidify. If the cultures failed to solidify, the gelatin was regarded as having been liquefied.

Cultures were grown in tubes of about 5 cc. of skim milk (tyndallized under vaseline) and also in citrated (transparent) milk (Brown and Howe, 1922). Gas formation, coagulation and peptonization were observed.

The digestion of various protein substances was observed by placing small bits of the test substances in tubes of veal bouillon under vaseline. The proteins used were 5 mm. cubes of coagulated horse serum or egg white and small masses of rennet casein and of washed horse or calf fibrin. These substances were autoclaved in the bouillon.

The attempt to determine the ability of various anaerobes to ferment various carbohydrates led to a number of interesting observations. The carbohydrates were sterilized in 10 per cent aqueous solutions and added aseptically to tubes of sterile bouillon under vaseline. Each tube also contained a bit of rabbit or guinea pig kidney which had been autoclaved in the bouillon. The tissue was added to provide conditions for a good growth of the culture. Plain veal infusion bouillon was used rather than fermented bouillon because the unfermented bouillon is a much better medium for the growth of some strains and the small amount of muscle sugar present was not sufficient to be detected with Benedict's solution or to result in the formation of enough acid to inhibit the growth of the culture or to be confused with the fermentation of larger amounts of the test substance added. It was very soon discovered that the customary practice of adding

1 per cent of carbohydrate to the medium was not to be relied upon for determining the fermentation reactions of anaerobes. Certain strains were found to ferment as much as 3 per cent of glucose. Although in media containing an excess of carbohydrate the final hydrogen ion concentration usually gave distinct evidence of fermentation it did not always do so. Even in media containing 4 per cent of glucose (always an excess) some glucose fermenting strains occasionally reached a final hydrogen ion concentration near that produced by non-fermenting strains (e.g., Botulinum A, an active fermenter of glucose, reached a final reaction of pH 6.2, whereas strains IV, Cf and Ci_1, non-fermenters, also reached reactions of pH 6.3, 6.2, and 6.3 respectively). The titratable acidity was found wholly unreliable as an index of fermentation. Certain strains incapable of fermenting glucose produced increases in titratable or reserve acidity of between 2 and 3 per cent normal acid (*e.g.*, IV, Ca, Ci_1, and Dc). In such cases there was also a large increase in the reserve alkalinity, both combining to make a large increase in the buffer index (Brown, 1921). An attempt was made to find an indicator which might be added to the test media so that the changes in reaction of the cultures might be followed. Litmus, neutral red, china blue and rosolic acid, reduced acid fuchsin, brom cresol purple, methyl red, phenol red, thymol blue and some others were tried but without exception all were decolorized by cultures of anaerobes whether fermentation occurred or not. Furthermore the color did not return to the indicators upon exposure of the culture to the air; they were permanently decolorized. Hall (1921) encountered the same difficulties in determining the fermentation reactions of anaerobes. Hall and Randall (1922) found cultures of *C. welchii* to reach a peak of acidity followed by a depression in the hydrogen ion concentration. Hall came to the conclusion that an increase in hydrogen ion concentration furnishes the best evidence of fermentation by anaerobes. To follow the changes in reaction that occurred in growing cultures of anaerobes a method for determining the hydrogen ion concentration of small amounts of fluid was devised (Brown, 1924). Peaks of acidity were noted to occur at different times in the case

of different anaerobes. With *C. welchii* the peak occurred within 48 hours. With other anaerobes it might occur at the end of a week or two, sometimes later. The results were usually but not always clear cut. Defining fermentation as synonymous with saccharolysis it would seem that the best evidence of fermentation would be the disappearance of the test substance from the culture. We therefore adopted the method of adding only 0.2 per cent of the carbohydrate to the bouillon and after incubation for three months or longer testing for the presence or absence of the carbohydrate. Eight or 10 drops of bouillon containing 0.2 per cent of glucose, maltose, or lactose gave a distinct reaction with about 4 cc. of Benedict's solution. By adding 1 or 2 drops of dilute hydrochloric acid to 8 or 10 drops of the sucrose bouillon and boiling it for a few seconds it also gave a positive reaction with Benedict's solution. The presence of starch was readily detected by the addition of Lugol's solution. If cultures in any of the above media were found to give negative chemical tests it was concluded that the carbohydrates had been fermented. Incubated controls always gave positive chemical tests. Mannitol, glycerol, and salicin were also added to bouillon in a concentration of 0.2 per cent, but simple chemical tests for these substances were lacking. A biological test was therefore tried.

A strain of the *B. coli* group was found to ferment these substances with gas production. Smith and Smith (1920) found that if strains of *B. suipestifer* were grown in lactose bouillon, *B. coli* was no longer able to form gas when superinoculated into these cultures, but that if the hog cholera culture in lactose bouillon was heated at or above 80°C. the gas inhibiting factor was removed and *B. coli* was able to form gas in the culture. Our method was as follows: After incubating the anaerobe cultures for a month or longer a determination of the hydrogen ion concentration was made and, if necessary, the reaction was adjusted to about pH 7.0 by the addition of alkali or acid. The culture was then heated in a boiling water bath for fifteen or twenty minutes, cooled, and superinoculated with *B. coli*. Good growth of the colon bacillus always occurred. Because of the high buffer index of many of the anaerobe cultures and the small amount of

carbohydrate originally present (0.2 per cent) a marked increase in the hydrogen ion concentration due to fermentation by *B. coli* was not to be expected, but the formation of an appreciable amount of gas was interpreted as indicating the presence and fermentation of the test substance. *B. coli* in control tubes of medium in which anaerobes had not grown and which contained 0.2 per cent of test substance always produced considerable gas. On the other hand the absence of gas formation by *B. coli* in the old anaerobe cultures had to be interpreted with caution because in some anaerobe cultures containing glucose, maltose, sucrose, or lactose as revealed by positive Benedict tests, *B. coli* failed to produce gas. We offer no explanation of the phenomenon but it would appear that the gas inhibiting factor is not always thermolabile as Smith and Smith found it to be in *B. suipestifer* cultures. In view of the difficulties encountered the results of the fermentation tests for mannitol, glycerol, and salicin are not reported in this paper.

One other qualitative test requires explanation—the hydrolysis of milk fat. The ability of certain bacteria to hydrolyze fat has been noted by a number of authors referred to in the paper by Orcutt and Howe (1921). No systematic use seems to have been made of this differential character for the differentiation of anaerobes. For this purpose we have employed the cream of cow's milk. Thick cream was washed several times with salt solution and recovered by centrifugation. A thick emulsion of the washed cream was made in salt solution and sterilized in the autoclave. If this is done carefully the emulsion is not destroyed. About 0.5 cc. of this emulsion was then pipetted aseptically into 5 cc. of sterile bouillon under vaseline. In time the cream rises to the surface forming a compact layer 1 to 2 mm. thick just beneath the vaseline. Hydrolysis of the fat was manifested by more or less complete transformation of the cream into a thick (sometimes 10 mm. or more) layer of gray flocculent material sometimes sinking to the bottom of the medium when disturbed. Chemical analysis of this material by Dr. Paul E. Howe showed it to consist largely of fatty acids or soaps, doubtless depending upon the reaction of the culture fluid.

To compare some of the products of growth of the anaerobes in plain bouillon with those formed in the presence of an excess of glucose each strain was inoculated into the following media: a tube containing 5 cc. of veal bouillon plus 0.5 cc. of distilled water, and a tube of 5 cc. of the same lot of bouillon plus 0.5 cc. of a 40 per cent glucose solution. The water and the glucose solution were added aseptically. Each tube contained also a small bit of rabbit or guinea-pig kidney to promote growth of the more delicately growing strains. The medium in each tube was covered by a layer of sterile vaseline 5 to 7 mm. thick. Sterile control tubes of each medium were incubated along with the inoculated tubes and were subjected to the same chemical analysis at the end of the period of incubation. The cultures were incubated for three months or longer if there was any evidence of continued activity. From time to time during this period the gas formed was measured and analyzed by the methods previously described (Brown, 1922). After each gas analysis the vaseline seal was melted and allowed to fall upon the surface of the medium. An effort was made to compare the composition of the first gas formed with that given off later. Frequent determinations of hydrogen ion concentration were also made. At the end of the period of incubation a few drops of the plain bouillon culture were tested for indol by the dimethylaminobenzaldehyde reaction and a few drops of the glucose bouillon culture were tested for sugar with Benedict's reagent to make sure that there was still an excess of unfermented sugar present. From the contents of each tube there were also made buffer index (pH 5.0 to 8.0) titrations, formol titrations and ammonia determinations. In the course of making these determinations there were also recorded the hydrogen ion concentration and the reserve or titratable acidity. The routine was as follows. Into each of four large (25 mm. diameter) test tubes there was accurately measured with an Ostwald pipette 1 cc. of the culture. To each tube was added 9 cc. of distilled water. Tubes I and II were used for the titration of the reserve acidity (RH) to an end point of pH 8.0 and then for the formol titration (Brown, 1923). Tubes III and IV were of Pyrex glass and were used first for the titration of the

reserve alkalinity (ROH) to an end point of pH 5.0 and then for ammonia determinations. The latter were made as follows. To each tube were added 3 or 4 small quartz pebbles, a small amount of powdered talc, a couple of drops of mineral oil and 1 cc. of potassium carbonate and potassium oxalate mixture (15 per cent of each). The ammonia was then distilled over a micro burner into dilute hydrochloric acid containing methyl red as an indicator. The unneutralized hydrochloric acid was titrated against N/20 sodium hydrate solution. The difference obtained by subtracting the ammonia from the formol titration is tabulated (table 3) as Amino Acid though it is to be understood as representing all formol titrating substances except the ammonia.

DISCUSSION OF RESULTS

The most conspicuous morphological feature of the anaerobes is the sporangium; the form and position of the spore and its size with respect to the cell. There are spherical spores and oval or elongated spores. The only strains with truly spherical spores encountered in this study were *Tetanus S. 304* and *Tetanomorphum C. 3413*. The spores of these strains were of course terminal and in size several times the diameter of the bacillus. Next to these, morphologically, stood the strain of *C. putrificum* received from Dr. Rettger. This organism had an almost spherical terminal spore terminating a delicate slender bacillus. The oval spores of the remaining strains were placed terminally, subterminally, or excentrically. No truly centrally placed spores were found. The relative size or diameter of the spore with respect to the thickness of the bacillus bore a general relationship to the position of the spore. Without exception the terminal spores were relatively large and the bacilli were long, delicate and slender. Sub-terminal spores were relatively a little smaller and the bacilli thicker. Excentrically placed spores were associated with rather thick rods, the spore sometimes expanding the cell very little. Of fifteen strains with terminal spores all were motile.

Hemolysis. A majority of the strains studied produced beta hemolysis in the blood agar plate (Brown, 1919). The deep

colonies were surrounded by clear, colorless, corpuscle-free zones. A minority of the strains were of the gamma type in blood agar, producing no visible change in the blood agar surrounding the colonies. None of the strains produced the alpha appearance. This is not surprising in view of the fact that the green-producing streptococci and pneumococci also fail to produce alpha zones under anaerobic conditions. Apparently oxygen is necessary for the production of the alpha zone. Strain Cf did produce a slight amount of greenish discoloration in the blood agar plate, but the green color appeared only after the anaerobe jar had been opened a second time for examination of the plates. It may be that if certain strains could be trained to grow aerobically they would produce alpha zones.

It is a striking fact that with the exception of *C. tetani* none of the terminal spore bearers produced hemolysis. It is especially to be noted that *Tetanomorphum C. 3413* was non-hemolytic. On the other hand all strains with sub-terminal or excentrically placed spores produced beta zones in blood agar, as did also *C. tetani*.

In the case of five strains, spores were not seen, although resistance to heat indicated that spores were produced by four of them. Of these strains four produced beta hemolysis; three of these were identified as strains of *C. welchii* and one as *C. chauvoei* known to have excentric or sub-terminal spores. The remaining strain in which spores were not seen was of the gamma type in blood agar, showing under the microscope slender and actively motile bacilli —all characters corresponding with those of terminal spore producers.

Colonies. Two distinct types of deep colonies were noted, compact biconvex colonies, and more or less diffuse rhizoid or woolly colonies. The most reliable means of determining the form of the deep colonies is in deep agar shake cultures after incubation for three or four days. The blood agar plates were usually incubated forty-eight hours. At this time the deep colonies of some strains were of a conglomerate or compound type or biconvex with satellites, but in deep agar shakes after longer periods of incubation these same strains produced woolly or rhizoid colonies.

Strains producing simple biconvex colonies in agar shake cultures always produced discrete round or scalloped surface colonies on blood agar plates and were never surface spreaders. Some of those producing woolly deep colonies also produced discrete surface colonies but most of them produced rapidly spreading amoeboid, rhizoid or filamentous surface films. *C. tetani* was one of the most persistent surface spreaders. The form of colonies could not be correlated with motility, form or position of spore, or with hemolysis.

Staining. All of the strains were Gram-positive when stained with Stirling's gentian violet although some strains were not strongly Gram-positive or were definitely Gram-positive only in young cultures. Immature spores retained the Gram stain. Mature spores were not stained. It was not uncommon to obtain Gram-positive vegetative cells and Gram-negative sporangia containing Gram-positive immature spores.

Lipolysis. Fat digestion, though a definite property of many strains, could not be correlated with any other character, morphological or physiological. Certain lipolytic strains were of a fermentative character and others were predominantly proteolytic. Strains which were both highly proteolytic and saccharolytic were also lipolytic, but *C. tetani* which is neither highly proteolytic nor fermentative was actively lipolytic whereas *Tetanomorphum C. 3413* was not lipolytic. Ability to hydrolyze fat is, therefore, a character which may be of considerable differential value within any large group of anaerobes, though it does not serve to distinguish one group from another.

Growth in milk. Results obtained in skim milk were generally paralleled by those in transparent (citrated) milk except that in the case of one or two strains growth was a little more abundant and more apparent in the latter. Reference to table 1 will show that milk was coagulated by most of the strains which failed to ferment lactose in bouillon. The acidity reached by these milk cultures was between pH 6.2 and 6.8. The coagulation in such cultures was a late coagulation, sometimes occurring during the second month of incubation. The degree of acidity was hardly sufficient to cause coagulation. On the other hand some of the

TABLE 1

Growth in cooked meat and in milk

STRAINS	GROWTH IN COOKED MEAT							MILK			FERMENTATION OF LACTOSE
	Gas formed				Action on meat			Gas	Coagulation	Peptonization	
	Total gas	Per cent CO_2			Color	Digestion	H_2S				
		First	Median	Last							
I, Ff, Cc, Ce, Ch, Fe, Dd	0.1–0.6	4–24		23–28	Pink to red	–	–	0.0–2.0	+	–	+
Chauvoei (Denver)	0.3				Pink	–	–	0.0	–	–	+
Chauvoei (Texas)	tr.				Pink	–	–	No growth			
Welchii X, Clf. 993, Ib, Ic	1.2–2.5	21–23		39–42	Pink to red	tr.	tr.	1.7–3.8	+	–	+
Putrificum	0.7	25	77	76	Pink to brown	+	+	Growth doubtful			+
Ci_2	1.3	66	97	68	Dark brown	+	+	0.6	+	+	+
Septique (Iowa)	0.5			24	Pink	–	–	0.2	+	–	+
Septique (Wash.)	0.2				Red	–	–	0.9	+	–	+
Cg	0.2			40	Light grey	+	±	0.4	+	+	–
III	1.6	40		99	Dark grey	+	+	0.6	+	+	±
II	1.6	85		99	Brown	+	+	1.1	+	+	+
Tetanomorphum (C 3413)	0.2				Pink	–	tr.	0.0	–	–	–
Carno-foetidum	2.0	87	92	42	Brown to black	+	+	0.8	+	+	–
Sporogenes H, Botulinum A, Botulinum B, C 710 Fa, Fd	1.3–1.9	20–85	95–100	45–99	Brown to black	+	+	0.3–0.9	+	+	–

Tetani S. 304	0.6		65		Pink	−	tr.	0.0	+	−	−
Fb	0.3		100	74	Grey to brown	+	+	0.4	+	+	−
Fa	0.3		88		Pink to brown	+	+	0.0	+	+	−
Cb, Ia, C. 710 Fb	1.3-3.0	87	99-100	81-100	Brown	+	+	0.0-1.2	+	+	−
C. 710 Fe, Fc, Ca, Histolyticum, Cd, IV	0.7-2.8	52-90	99-100	87-97	Red to brown	+	+	0.0-1.4	+	+	−
Ci_1, Cf, Da, Dc	0.0-0.5			83	Pink to brown	+	+	0.0	±	±	−

Explanation. The transverse rulings serve to divide the strains into the "metabolic groups" defined in table 2.

Gas is measured in volumes. See explanation under table 3.

tr. = trace.

Under per cent CO_2: First = during the first part of the period of gas formation; Median = during the median period of gas formation; Last = at the end of the period of gas formation.

Figures separated by a dash (e.g., 0.1–0.6) indicate minima and maxima for the strains of the group.

whey from these cultures transferred to fresh raw milk failed to cause coagulation within two or three hours at 37°C. so that if an enzyme was the cause of coagulation it must have become inactivated by the time the test was made.

Biological grouping of the anaerobes

All bacteria have a nitrogenous metabolism. Most of them attack amino acids, polypeptides, peptones, and probably albumoses and to this extent may be said to be proteolytic, but some bacteria attack also higher native protein substances such as were used in our tests, i.e., gelatin, casein, serum, egg white, and fibrin. The term proteolytic is usually reserved for those organisms which are able to attack the higher native protein substances.

Henry (1917) and others have grouped the anaerobes as saccharolytic, proteolytic, or as belonging to an intermediate group of organisms which are neither very proteolytic nor saccharolytic. This grouping has been very useful, but most anaerobes are both proteolytic and saccharolytic and although one or the other activity may be the more conspicuous the other is not to be ignored. The group of anaerobes we have studied, comprising many non-pathogenic as well as certain pathogenic strains, is probably more comprehensive biologically than would be a collection of strains from war wounds, and although this report does not include as many strains as might be desired it is believed that it furnishes the outline of a natural grouping which is illustrated in table 2. By noting the action of the anaerobes on a few protein substances and a few common carbohydrates there appears to be what E. C. Howe (1912) called a "metabolic gradient" with respect to each group of substances. Of the protein substances it is found that all the strains studied produced an increase in formol titration and in ammonia in pepton bouillon indicating that they attack amino acids and other low protein derivatives. Some strains could also liquefy gelatin; some could digest casein; some could digest coagulated albumin (egg white or serum); and others could also digest fibrin. Any strain which could digest fibrin was able to digest all the other substances in the series. Any that could digest coagulated albumin could also digest casein and

gelatin. Any that attacked casein also liquefied gelatin. We have therefore a series of protein substances listed in the order of their digestibility by anaerobes. The carbohydrate series is not quite so rigid. The significant carbohydrates for determining the major groups are glucose, lactose, sucrose, and starch. Other substances may be useful in sub-dividing some of the major groups. Action on maltose always ran parallel to that on glucose. Glucose was naturally the lowest member of the series and was fermented by all strains capable of attacking any of the carbohydrates. Starch was the highest member of the series. Any strain fermenting starch was also able to ferment glucose and lactose or sucrose or both of the disaccharides. We did not encounter *C. oedematiens* or *C. novyi* which are said to ferment glucose and starch but neither lactose nor sucrose. In table 2, therefore, each strain listed is to be understood as attacking all the proteins listed to the left of its place on the chart but not those to the right, and all the carbohydrates beneath it unless otherwise indicated by a negative sign, but not those above it. For example, strain Cd attacks all of the protein substances but none of the carbohydrates, whereas Fe attacks all of the carbohydrates (except sucrose) but none of the proteins above proteoses. For descriptive convenience and accuracy we shall refer to the position of a strain in the table by naming the highest members of the carbohydrate and protein series which it attacks. For example, Fe is a starch-proteose organism, Cd a non-saccharolytic-fibrin strain. In a rough sense, *C. vibrion-septique* may be said to belong to a "saccharolytic" group, *C. sporogenes* to a "proteolytic" group, and *C. tetani* to an "intermediate" group, but to which group does strain Ci_2 belong? It is more saccharolytic than *C. vibrion-septique* and as proteolytic as *C. sporogenes* and in both respects is unlike *C. tetani*. If, however, it is said that *C. vibrion-septique* belongs to the lactose-gelatin group, *C. sporogenes* to the glucose-fibrin group, *C. tetani* to the non-saccharolytic-gelatin group, and Ci_2 to the disaccharide-fibrin group, their metabolic positions are pretty well defined. If desired, many of the groups may be designated by the names of well known members.

TABLE 2

Grouping of the anaerobes in accordance with their saccharolytic and proteolytic powers

SACCHAROLYTIC GROUP	STRAIN	SPORE: Form and position	MOTILITY	FORM OF DEEP COLONY	TYPE IN BLOOD AGAR	LIPOLYSIS
Starch	I	o., t.	+	e	γ	+
	Fl	o., t.	+	e	γ	+
	Ce	o., t.	+	e	γ	+
	Ce	o., t.	+	e	γ	−
	Ch	o., t.	+	e	γ	±
	Fe (sucrose −)	o., t.	+	e	γ	+
	Dd (sucrose)	o., t.	+	e	γ	±
Sucrose and lactose						
Sucrose or lactose						

SACCHAROLYTIC GROUP	STRAIN	SPORE: Form and position	MOTILITY	FORM OF DEEP COLONY	TYPE IN BLOOD AGAR	LIPOLYSIS
Starch	Chauvoei (Denver)	o., exc.	±	c	β	+
	Chauvoei (Texas)		+	e	β	+
Sucrose and lactose						
Sucrose or lactose	Septique (Iowa) (suc. −)	o., s-t., exc.	+	d	β	+
	Septique (Wash.) (suc. −)	o., s-t., exc.	+	d	β	+

SACCHAROLYTIC GROUP	STRAIN	SPORE: Form and position	MOTILITY	FORM OF DEEP COLONY	TYPE IN BLOOD AGAR	LIPOLYSIS
Starch	Welchii X (capsules +)	o., exc.	−	c	β	±
	Clf. 993		−	c	β	+
	Ib (capsules +)		−	d	β	±
	Ic (capsules +)		−	d	β	+
Sucrose and lactose						
Sucrose or lactose	Cg (lactose−)	o., exc.	−	d	β	−

SACCHAROLYTIC GROUP	STRAIN	SPORE: Form and position	MOTILITY	FORM OF DEEP COLONY	TYPE IN BLOOD AGAR	LIPOLYSIS
Starch						
Sucrose and lactose						
Sucrose or lactose	III (lactose ±)	o., s-t.	+	d	β	+
	II (sucrose ±)	o., s-t., exc.	−	d	β	+

SACCHAROLYTIC GROUP	STRAIN	SPORE: Form and position	MOTILITY	FORM OF DEEP COLONY	TYPE IN BLOOD AGAR	LIPOLYSIS
Starch	Putrificum	o., t.	+	d	γ	+
Sucrose and lactose	Cl_2	o., s-t., exc.	+	d	β	+
Sucrose or lactose						

| Proteolytic group | | |

The starch-proteose group is the *sphenoides-tertius* group.
The starch-gelatin group is the *chauvoei* group.
The starch-casein group is the *welchii* group.
The lactose-gelatin group is the *vibrion-septique* group.
The glucose-fibrin group is the *sporogenes-botulism* group.
The non-saccharolytic-gelatin group is the *tetanus* group.

The non-saccharolytic-fibrin group may be called the *histolyticus* group though it evidently contains two distinct sub-groups. To this group *C. putrificum* would also belong were it not for the fact that it has some action on carbohydrates. This species can not be dismissed as a mixed culture as was suggested by the British Medical Research Committee (1919). The strain received from Dr. Rettger had every appearance of being pure and a distinct entity. We plated it out many times. The morphology was exactly like the illustration in the paper of Reddish and Rettger (1922). By careful measurement the terminal spores were found to be almost but not quite spherical. The very slow delicate growth and the proteolytic activity were exactly as described by Reddish and Rettger. Although these authors describe *C. putrificum* as very slightly or not at all saccharolytic they did find that it was able to destroy an appreciable amount of glucose. In our study we have noted the disappearance of small amounts not only of glucose but also of lactose, sucrose and starch, so that we regard the organism as having rather moderate but broad fermentative powers. In our grouping it belongs to the starch-fibrin group although its metabolism is carried on at a very slow rate. Cultures were sometimes incubated for 2 weeks before there was gross evidence of growth. In one important respect our results differ from those of Reddish and Rettger. We find *C. putrificum* a copious gas former if given sufficient time under the right conditions. The cultures were grown in vaseline tubes and the gas produced was measured in terms of the volume of medium. The course of gas production in cooked meat (horse or beef heart) medium was as follows:

Incubated 2 weeks—small bubble of gas
Incubated 4 months—large bubble of gas, 25 per cent CO_2
Incubated 7 months—0.7 volume of gas, 75 per cent CO_2
Incubated 13 months—1.9 volume of gas, 79 per cent CO_2

Sub-cultures were made from the above culture at the end of four months and thirteen months of incubation and each of these went through the same slow course of development and gas formation. There was no evidence of contamination. In plain bouillon plus a bit of guinea pig kidney 0.3 volume of gas was produced in four months and the final pH was 7.0, but in the same medium plus 4 per cent of glucose 2.8 volumes of gas were produced in four months and the final reaction was pH 5.9 (see table 3). Gas formation was also increased and accelerated in bouillon containing maltose, lactose, sucrose, starch, mannitol, glycerol, and salicin.

With regard to other than saccharolytic and proteolytic characters some of the groups show a degree of uniformity which is encouraging to one who has frequently been discouraged by the apparent chaos among the sporulating anaerobes. All the members of the starch-proteose group studied are slender motile bacilli producing oval terminal spores, and rather compact colonies of the gamma type in blood agar. They differ with respect to the hydrolysis of fat. Members of the starch-gelatin and starch-casein groups produce beta hemolysis in blood agar, are non-motile (*C. welchii*) or slightly motile (*C. chauvoei*), produce sub-terminal or excentric oval spores, generally form compact colonies and generally hydrolyze milk fat. Members of the glucose-fibrin group also produce beta hemolysis, have sub-terminal or excentric oval spores, are motile, form diffuse colonies, and actively hydrolyze milk fat. Members of the non-saccharolytic-albumin group and the non-saccharolytic-fibrin group include at least two varieties, an oval terminal spore bearer, lipolytic but non-hemolytic, and a sub-terminal spore bearer, non-lipolytic but hemolytic. All of the well known pathogens produce beta hemolysis in blood agar and hydrolyze milk fat. The morphological and gross cultural characters serve to group the strains as shown in table 2.

BIOCHEMICAL STUDY

A more detailed biochemical study was made of cultures grown in plain infusion bouillon and in the same medium plus 4 per cent of glucose, each tube containing also a bit of rabbit or guinea

TABLE 3

The biochemical analysis of cultures in plain bouillon and in glucose bouillon

STRAINS	FINAL pH	RESERVE ACIDITY		BUFFER INDEX		CHANGES IN			INDOL	GAS ABOVE THE MEDIUM				CARBON DIOXIDE		
		Actual	Increase	Actual	Increase	Formol titration	Ammonia	Amino acids		Total	Per cent CO_2			Above the medium	In the medium	Total
											First	Median	Last			
I, Ff, Ce, Ce, Ch, Fe, Dd	5.9–6.6	1.2–2.6	0.3–2.0	3.9–5.3	0.4–1.7	0.9–4.2	0.9–1.4	−0.3–3.3	+	tr.–0.2					0 0 0.5	0 0 0.5
	5.2–4.7	*3.8–6.4*	*2.7–5.4*	*3.8–6.8*	*0.1–2.9*	*−0.2–3.1*	*0.0–0.6*	*−0.5–3.1*		*0.5–1.6*	*26–38*		*50–41*	*10.1–0.6*	*0 0–0.2*	*0.1 0.8*
Chauvoei (Denver)	7.0	1.0	0.1	4.0	0.5	0.6	1.0	−1.3	−	0.0					0.2	0.2
	6.0	*3.2*	*1.1*	*4.0*	*0.1*	*1.0*	*1.0*	*0.0*		*0.5*	*18*		*30*	*0.1*	*0.2*	*0.3*
Chauvoei (Texas)	7.1	1.7	0.8	6.6	3.1	4.2	6.2	−2.0	−	0.0					0.4	0.4
	6.0	*3.8*	*1.2*	*4.4*	*0.5*	*0.8*	*1.3*	*−1.1*		*0.1*				*tr.*	*0.1*	*0.1+*
Welchii X, Clf. 993, Ib, Ic	7.3–6.8	1.2–1.4	0.3–0.7	4.1–4.7	0.7–1.2	1.0–6.1	2.0–2.4	−0.4–3.9	+	0.2–0.3					0 2–0.4	0 2–0.4
	4.9–4.6	*5.1–6.8*	*4.8–5.8*	*4.7–6.0*	*1.8–3.0*	*0.5–1.3*	*0.3–0.6*	*0.1–1.1*		*2.3–2.5*	*36–41*	*47*	*40–51*	*0.90–1.1*	*0 0–0.1*	*0 9–1.2*
Putrificum	7.0	2.1	1.1	10.9	7.2	6.2	9.0	−2.8	+	0.3				0.1	1 0	1.1
	5.9	*3.6*	*1.7*	*7.8*	*3.0*	*4.6*	*5.0*	*−1.4*		*2.8*	*57*		*60*	*1 6*	*0.6*	*2.2*
Cis	7.0	1.5	0.7	8.2	4.7	4.9	6.9	−2.0	tr.	tr.					0.8	0 8
	5.1	*4.8*	*2.8*	*7.1*	*3.1*	*4.4*	*2.0*	*2.4*		*3.7*	*93*	*76*	*89*	*3.2*	*0 5*	*3 7*
Septique (Iowa)	7.1	1.5	0.6	5.0	1.5	1.8	1.3	0.5	−	0.2				tr.	0.3	0 3+
	5.6	*5.4*	*3.4*	*6.5*	*2.6*	*5.3*	*1.6*	*3.8*		*2.1*	*54*	*43*	*56*	*0 8*	*0.2*	*1.0*
Septique (Wash.)	7.0	1.2	0.4	6.8	3.3	2.7	4.9	−2.2	−	tr.					0.4	0.4
	5.5	*4.5*	*2.5*	*5.5*	*1.6*	*2.6*	*1.3*	*1.3*		*2.5*	*54*	*42*	*48*	*1.0*	*0.2*	*1 2*
Cg	6.9	1.9	1.1	6.6	3.1	4.3	6.2	−1.9	+	tr.					0 4	0.4
	5.7	*4.5*	*2.6*	*6.9*	*3.0*	*4.0*	*5.0*	*1.0*		*3.5*	*57*	*69*	*57*	*1 8*	*0 5*	*2.3*
III	7.2	1.3	0.6	8.2	5.1	9.8	8.4	1.4	tr.	0.1				0.1–	0 7	0.8
	5.5	*4.2*	*2.5*	*6.0*	*2.3*	*6.7*	*3.7*	*3.0*		*5.7*	*61*	*89*	*63*	*4.5*	*0 0*	*4.5*
II	7.0	1.1	0.4	8.5	5.3	11.0	8.8	2.2	+	0.2				0.1	0 7	0.8
	5.3	*4.5*	*2.8*	*6.1*	*2.3*	*7.2*	*3.4*	*3.8*		*4.5*	*92*	*100*	*71*	*4.0*	*0 1*	*4.1*

Tetanomorphum (C. 3413)	0.9	1.3	0.3	5.6	1.9	1.3	0.5	0.8	−	tr.					0.4	0.4
	5.7	*3.6*	*1.9*	*5.8*	*1.6*	*0.9*	*0.6*	*0.4*		*0.1*			*19*		0.4	0.4
Carno-foetidum	7.1	1.5	0.5	8.8	5.2	7.9	8.2	−0.3	+	0.0					1.1	1.1
	5.6	*4.5*	*2.0*	*6.7*	*2.5*	*5.3*	*1.9*	*3.4*		*4.1*	*71*	*94*	*81*	*3.6*	*0.5*	*4.1*
Sporogenes H, Botulinum A, Botulinum B, C. 710Fa, Fd	7.1–6.8	1.4–2.1	0.4–1.5	7.3–8.8	3.7–5.2	5.2–9.4	5.6–7.9	−2.0–2.3	−	0.0 tr.					0.7–1.1	0.7–1.1
	6.2–5.3	*3.1–5.6*	*1.2–3.9*	*6.4–7.3*	*3.1–3.5*	*2.9–7.3*	*1.2–2.6*	*0.5–5.1*		*3.4–3.1*	*44–93*	*89–90*	*66–95*	*2.9–7.6*	*0.4–0.8*	*3.4–8.2*
Tetani S. 304	7.3	0.7	−0.1	6.7	3.2	3.1	4.8	−1.7	−	tr.					0.8	0.8
	6.7	*2.1*	*1.0*	*8.0*	*4.1*	*5.9*	*5.0*	*0.9*		*0.2*			*50*	*0.1*	*1.3*	*1.4*
Fb	6.7	1.5	0.8	6.3	3.2	7.4	6.8	0.6	+	tr.					0.5	0.5
	6.5	*2.5*	*0.9*	*6.4*	*2.7*	*6.5*	*6.6*	*−0.1*		*0.1*			*55*		*0.6*	*0.6*
Fa	7.0	1.9	1.3	7.0	3.4	7.8	6.9	0.9	tr.	0.0					0.7	0.7
	6.0	*2.6*	*1.6*	*7.2*	*3.3*	*7.1*	*5.9*	*1.2*		*0.0*					*0.7*	*0.7*
Cb, Ia, C. 710Fb	7.0–6.7	1.8–1.9	0.9–1.1	7.8	4.1–4.7	6.3–11.3	7.0–11.2	−0.7–0.3	−	0.0–0.1					0.6–0.7	0.6–0.7
	7.0–6.6	*2.3–3.1*	*0.7–1.9*	*7.6–8.7*	*3.8–4.9*	*8.6–10.0*	*7.6–9.9*	*0.1–0.3*		*tr.–0.2*			*40–60*	*0.1*	*0.6–0.7*	*0.7–0.8*
C. 710Fe, Fc, Ca, Histolyticum, Cd, IV	7.0–6.5	1.9–3.5	1.2–2.8	7.3–9.5	4.2–6.4	8.4–12.2	8.5–11.0	−0.1–1.2	*	0.0–0.1					0.6–0.8	0.6–0.8
	7.0–6.3	*2.2–3.8*	*0.5–2.2*	*7.2–9.3*	*3.5–5.6*	*8.5–10.4*	*7.9–9.2*	*−0.4–1.9*		*tr.–0.3*			*40–76*	*0.1–0.2*	*0.5–0.8*	*0.6–1.8*
Ci, Cf, Da, Dc	7.0–6.7	2.0–3.7	1.1–2.8	7.8–9.9	4.3–6.4	5.2–10.4	8.6–13.5	−6.0–0.3	=	0.0–tr.					0.4–0.7	0.4–0.7
	6.9–6.2	*2.5–3.7*	*1.3–3.2*	*7.4–8.6*	*3.6–5.6*	*4.0–10.5*	*6.8–10.1*	*−3.1–0.1*		*0.0*					*0.5–0.7*	*0.5–0.7*

Explanation. The transverse rulings serve to divide the strains into the "metabolic groups" defined in table 2.

Figures separated by a dash (e.g., 1.2–2.6) indicate minima and maxima for the strains of the group. Figures in Roman type are for cultures in plain veal infusion bouillon. Figures in italics are for cultures in veal infusion bouillon plus four per cent of glucose.

The reserve acidity is the titratable acidity to an end point of pH 8.0.

The buffer index represents the amount of acid or alkali required to change the reaction of the culture from pH 5.0 to 8.0 or vice versa. The results of titration are in terms of per cent normal acid or alkali.

Figures under formol titration, ammonia and amino-acids indicate increases or decreases (when preceded by a minus sign) as compared with titrations of sterile incubated controls of the same media. The results are in terms of per cent normal. By multiplying these figures by 14 the results may be reduced to milligrams of nitrogen per 100 cc. of culture.

Amounts of gas above the medium and of carbon dioxide above and in the medium are expressed as volumes, e.g., 5 cc. of gas measured at atmospheric pressure and at incubator temperature, when given off by 5 cc. of culture equals 1.0 volume of gas.

Under Per cent CO_2: First = during the first of the period of gas formation; Median = during the median period of gas formation; Last = at the end of the period of gas formation.

Numerical values of less than 0.1 are not reported.

pig kidney (autoclaved) and a covering of vaseline. The biochemical results are tabulated in table 3. Although by the analytical methods employed duplicate determinations were found to give almost identical results when made with samples of the same culture, the results in table 3 are not to be read too closely. The biological factor is quite large and the metabolism of the anaerobic bacteria is very complex. If the same organism is grown in two or more tubes of the same medium, the results of chemical analysis may show not an actual divergence but considerable quantitative variation. The factors which influence the growth of the culture are not all understood or under control. It would appear that under certain conditions the proteolytic activity of an anaerobe may be most conspicuous, while under slightly different conditions the fermentative activity may be in the ascendency. It is necessary to interpret the results rather broadly and to emphasize general tendencies rather than minute differences.

Members of the starch-proteose group and those of the starch-casein group resembling *C. welchii* are readily recognized as fermenters of glucose. In 4 per cent glucose bouillon they produce a fairly high hydrogen ion concentration (pH 4.6 to 5.2) accompanied by a pronounced increase in reserve or titratable acidity and the evolution of a fair amount of gas. Botulinum A of the glucose-fibrin group produced a final hydrogen ion concentration of pH 6.2 in 4 per cent glucose bouillon, accompanied by only a fair increase in reserve acidity but a very large amount of gas. It consumed a large amount of glucose. Strain Cf also produced a final hydrogen ion concentration of pH 6.2 in 4 per cent glucose bouillon but the reserve acidity was very little above that of a plain bouillon culture and only 0.1 volume of gas was produced. There was no appreciable consumption of glucose and all the available data indicate that this strain does not ferment glucose. Strains Ca and Ci_1 when grown in plain bouillon had a titratable acidity of 3.5 and 3.7 per cent normal respectively, or 2.8 above their sterile controls. However, their hydrogen ion concentrations were only pH 6.6 and 6.7 respectively, and in glucose bouillon they produced practically no gas and consumed no

appreciable amount of sugar. The explanation of the high titratable acidities is to be found in the marked increase in buffer substances. They showed marked increases in reserve alkalinity as well as in reserve acidity, and the production of large amounts of ammonia indicated the deamination of large amounts of amino acids resulting in the liberation of fatty acids as well as ammonia. It is evident that proteolysis may result in an increased titratable acidity which may be mistakenly held to indicate fermentation. The results of Kendall, Day and Walker (1922) illustrate the same phenomenon although they used titration as an index of fermentation.

Of the glucose fermenting organisms here reported Botulinum A produced the lowest final hydrogen ion concentration (pH 6.2) but the largest amount of gas (8.1 volumes) and in this culture consumed almost 4 per cent of glucose. Of the total gas produced 96 per cent was CO_2. A parallel culture of Botulinum B reached a hydrogen ion concentration of pH 5.3 and produced only 3.6 volumes of gas, of which 83 per cent was CO_2. Whether these differences are constant for other strains of Botulinum A and B has not been determined. It is noticeable, however, that all members of the sporogenes-botulinum group and the neighboring glucose-albumin group, when grown in glucose bouillon, consume large amounts of sugar, attain only a moderate degree of acidity (pH 5.3 to 6.2), and produce a large amount of gas (3.5 to 8.1 volumes), of which a large part (83 to 96 per cent) is CO_2. In contrast with this group is the tertius or starch-proteose group, which is almost purely fermentative in character. These organisms, when grown in glucose bouillon, actually consume less sugar than those of the sporogenes-botulism group but reach a higher acidity (pH 4.7 to 5.2) accompanied by less gas (0.3 to 1.6 volumes) of which only 26 to 36 per cent is CO_2. Evidently the percentage of CO_2 in the gas above the medium is not dependent wholly upon the acidity of the culture. Various hypotheses suggest themselves as possible explanations of the differences. Members of the sporogenes-botulinum group are highly proteolytic. They produce marked increases in the formol titrating substances of the media. In plain bouillon a large part of the

amino acids is deaminized. There is no apparent reason why the fatty acids liberated should not be further broken down with the liberation of gas. However, since very little gas was produced by any of the cultures in plain bouillon and since none of the non-fermenters produced more than a trace of gas, we must conclude that in glucose bouillon most or all of the gas is derived from the fermentation of the sugar. The large amount of gas produced by the sporogenes-botulinum group is doubtless related to their large sugar consumption. The large sugar consumption is permitted by the maintenance of a relatively low hydrogen ion concentration. The low hydrogen ion concentration is probably maintained by several factors; the marked increase in buffer index, largely the result of proteolysis; and the alkaline fermentation of organic acid salts as described for certain aerobic bacteria by Ayers and Rupp (1918). The formation of a high percentage of CO_2 by members of this group of anaerobes is strong evidence that the latter process is an important one. That proteolytic activity contributes to the same end, however, is indicated by the fact that if we compare the gas production of different proteolytic groups within the same fermentative group, the amount of gas produced and the percentage of CO_2 regularly increase with proteolysis; in fact by subtracting the volume of CO_2 from the total free gas produced it is found that the greater amount of total gas produced by the more proteolytic organisms is due entirely to CO_2.

Before we leave the study of gas production by the fermentative anaerobes another phenomenon is worthy of notice. The method of determining the CO_2 in the vaseline tubes made it possible to determine the percentage of CO_2 given off at various intervals during the period of gas formation. This was done in the case of glucose bouillon cultures and cooked meat cultures. In all cases it was found that the first gas given off contained a lower percentage of CO_2 than that given off during the middle period of gas formation, (noted also by Wolf and Harris, 1917, for *C. welchii*, and by Bushnell, 1922, for *C. sporogenes*), and in most cases the last gas given off also contained a lower proportion of CO_2. The increase in CO_2 may be the result of a rising acidity

but the subsequent drop in the percentage of CO_2 could not be explained by a change in reaction because in 4 per cent glucose bouillon a reversal of reaction did not occur.

It is not surprising that any of the fermenters of glucose should produce gas in cooked meat medium (see table 1), but it is surprising to find that the largest amounts of gas in this medium were produced by strains (e.g., Histolyticum Fc, Ca, Cb, Cd, and Ia) which did not ferment glucose or any of the test carbohydrates used. The same strains did not produce more than a few bubbles of gas in plain bouillon or glucose bouillon but produced gas in milk and gelatin as indicated in table 4. Less gas was produced in transparent citrated milk than in plain skim milk.

TABLE 4

Gas production by non-fermentative anaerobes

STRAIN	MEDIA				
	Plain bouillon	Glucose bouillon	Cooked meat	Milk	Gelatin
Fc	Trace	0.12	1.69	1.4	1.4
Ca	0.1	0.1	2.05	0.9	0
Cb	0	0.1	3.0	1.25	0
Cd	Trace	0.1	2.81	0.9	1.8
Ia	0.1	0.17	1.5	Trace	Trace
Histolyticum	Trace	Trace	1.0	0	2.0

Figures are in terms of the volume of medium used.

In all media the gas produced by these strains during the middle of the period of gas formation was 100 per cent CO_2. It is difficult to account for the gas formation by these non-fermenters of glucose unless they are able to attack certain lower carbohydrates or organic acids which may be present in these media but are absent from infusion bouillon. The acidity did not rise above pH 6.5 and in most cases was near pH 7.0.

The amount of CO_2 in solution in the media revealed nothing of recognized importance. All bacteria probably produce some CO_2; the result of what Hesse (1893) called the respiratory activity of bacteria. The amount of CO_2 found in solution bears a fairly consistent relation to (*a*) the amount of growth, (*b*) the hydrogen ion concentration of the culture fluid, and (*c*) the CO_2

tension of the gas above the medium. The amounts of CO_2 found in cultures of the anaerobes in plain and glucose bouillon are tabulated in table 3.

Differences in the metabolism of the non-saccharolytic and the saccharolytic anaerobes are quite evident. The non-fermenters produced very little gas in either plain or glucose bouillon. Although the hydrogen ion concentration of the glucose bouillon cultures was never above pH 6.2 it was usually a little higher than that of the plain bouillon cultures. This may be attributed in part to the fact that the incubated sterile control tubes of glucose bouillon were usually a little more acid than the plain bouillon.

The buffer indices of the more proteolytic anaerobes were higher than those of the less proteolytic strains. As might be expected, the formol titration usually ran roughly parallel with increase in buffer substance. The amount of NH_3 produced is an index of deamination but is not necessarily a measure of proteolysis as has been assumed by Kendall. The proteolytic process may progress as far as the production of amino acids without giving evidence in the form of increase in ammonia. Strain Fe (table 3) shows a tendency to do this. On the other hand the amino acids alone do not furnish a good index of proteolysis, as was assumed by DeBord (1923), because they may be more or less completely deaminized as was the case with all the non-fermentative anaerobes studied. However, the formol titration which includes ammonia, amino acids and the lower polypeptides is a very good index of proteolysis when any of these products are formed. The figure for the total ammonia determination was naturally never as large as that for the formol titration but the increase in the ammonia was often greater than the increase in the formol titration and could only indicate a decrease in amino acids and similar substances (e.g., Strains Cf and Ci_1).

In the case of the fermentative anaerobes generally the increase in formol titration of the plain bouillon culture was greater than that of the glucose bouillon culture, suggesting that the carbohydrate had a sparing action on the protein to some extent, sometimes greater than if the ammonia alone were taken as an

index of proteolysis (Ic, I, Fe, Ff, Cc, Ce, Dd, and Ib) but more often less (Ch, *C. welchii* X, Clf. 993, *C. chauvoei* Texas, Ci_1, *C. vibrion-septique* Washington, Cg, III, *C. sporogenes*, *Carno-foetidus*, II, and *Botulinum B*). In the latter cases one might say that the carbohydrate had spared amino acids to a greater extent than it had spared the protein. However, in the case of strains *C. vibrion-septique* Iowa, *Botulinum A* and *C. chauvoei* Denver, it might be concluded that the carbohydrate had stimulated proteolysis since the formol titration of the glucose bouillon cultures showed a greater increase than that of the plain bouillon cultures. We have also experimented with the effect of an excess of glucose on the digestion of certain protein substances by a saccharo-proteolytic anaerobe. *Botulinum B* was used for this purpose because it appeared to show a marked protein sparing action by glucose. The digestion of coagulated serum and of cooked meat was noticeably less in the presence of glucose, but the liquefaction of gelatin was not prevented. Other anaerobes gave similar results. All of the above results, however, must be interpreted with caution. If it were possible to study the metabolism of a single bacillus or of a constant number of active bacilli the meaning of the results might be quite clear, but in bacterial cultures there are the complicating factors of growth and activity. The accumulation of acids or other inhibitory substances may be responsible for the reduced nitrogenous metabolism. A glucose bouillon culture of a fermenting organism generally has the appearance of heavier growth than a plain bouillon culture but it may show a more rapid development and shorter life than the plain bouillon culture. It may be that the proteolytic activity of these cultures is proportional to the total amount of bacterial growth and activity and that the sparing action of the carbohydrate is only apparent. The problem calls for more careful control of all the possible factors involved.

SUMMARY

No markedly pathogenic or toxin-producing anaerobes were found in the intestinal tract of the calf studied.

Both proteolytic and saccharolytic anaerobes were found at all

levels of the intestinal tract but the putrefactive organisms were more abundant in the cecum and large intestine.

Of all the strains studied from the calf and from various other sources, none produced the alpha appearance in blood agar, a large majority produced beta hemolysis, and some produced the gamma appearance.

More than half the strains studied had the ability to hydrolyze the fat of cow's milk to soaps or fatty acids.

By noting the action of anaerobes on a few protein and carbohydrate substances it is found that each of these groups of substances may be arranged in a series in order of the ease with which the substances are attacked by anaerobes. Strains which attack the same carbohydrates and the same protein substances form "metabolic groups." Within each group there is a fairly satisfactory correlation of morphological, physiological and pathogenic characters so that the groups form the basis of a convenient and natural laboratory classification.

Some anaerobes produce profound changes in media within a few days. Some may complete their fermentative activity within a few days while their proteolytic activity may extend over several months. Others may produce perceptible fermentation or proteolysis only after two or more weeks and the activity may continue for several months. Whether the changes produced throughout these longer periods are due to actively growing organisms or to enzymes elaborated and released into the media has not been determined.

The titratable acidity is wholly unreliable for determining the occurrence or non-occurrence of fermentation by anaerobes. These organisms often produce large increases in the buffer index of media. The increase in titratable acidity is not necessarily accompanied by increase in hydrogen ion concentration and may occur in the absence of fermentation. In the presence of an excess of carbohydrate fermentation is usually indicated by an increase in hydrogen ion concentration but for many strains 1 per cent of carbohydrate is insufficient. A few cultures have been found to ferment as much as 3 per cent of glucose and arrive at a hydrogen ion concentration only a little above neutrality. The disappear-

ance of the carbohydrate from the medium is regarded as the best evidence of fermentation.

The chemical analysis of cultures grown in plain bouillon and in bouillon plus 4 per cent of glucose (always an excess) has shown that neither ammonia production nor amino-acid production is a good index of proteolysis but that the formol titration provides a much better index.

The results of gas measurement show that much more gas is produced, though more slowly, by some of the more proteolytic anaerobes than by *C. welchii*. Gas analysis shows that the proportion of CO_2 to other gases found above the medium is least at the beginning of gas formation, increases often to 100 per cent of the gas given off during the intermediate period, and again decreases towards the end of the gas-forming period. Whether the gas produced by anaerobes is all from carbohydrates or not remains an open question.

Although in cultures of anaerobes there is usually less proteolysis in the presence of an excess of fermentable carbohydrate many other factors must be taken into consideration before it can be said that the carbohydrate has "spared" the protein in the sense that it has altered the metabolism of the organisms.

REFERENCES

Adamson, R. S. 1919 On the cultural characters of certain anaerobic bacteria isolated from war wounds. Jour. Path. and Bact., **22**, 345.

Ayers, S. H., and Rupp, P. 1918 Simultaneous acid and alkaline bacterial fermentations from dextrose and the salts of organic acids respectively. Jour. Infect. Dis., **23**, 188.

British Medical Research Committee 1917, 1919 Reports.

Brown, J. H. 1919 The use of blood agar for the study of streptococci. Monograph of the Rockefeller Institute for Medical Research, No. 9, New York.

Brown, J. H. 1921 An improved anaerobe jar. Jour. Exper. Med., **33**, 677.

Brown, J. H. 1921 Hydrogen ions, titration and the buffer index of bacteriological media. Jour. Bact., **6**, 555.

Brown, J. H. 1922 The vaseline tube and syringe method of micro gas analysis of bacterial cultures. Jour. Exper. Med., **35**, 667.

Brown, J. H. 1922 A method for the separation of spore-bearing anaerobes from other spore-bearing bacteria. Jour. Lab. and Clin. Med., **7**, 692.

Brown, J. H., and Howe, P. E. 1922 Transparent milk as a bacteriological medium. Jour. Bact., **7**, 511.

BROWN, J. H. 1923 The formol titration of bacteriological media. Jour. Bact., **8**, 245.

BROWN, J. H. 1924 The colorimetric determination of the hydrogen ion concentration of small amounts of fluid. Jour. Lab. and Cli. Med., **9**, 239.

BUSHNELL, L. D. 1922 Quantitative determinations of some of the biochemical changes produced by a saprophytic anaerobe. Jour. Bact., **7**, 373.

DEBORD, G. G. 1923 Certain phases of nitrogenous metabolism in bacterial cultures. Jour. Bact., **8**, 7.

HALL, I. C. 1921 Criteria in anaerobic fermentation tests. Jour. Infect. Dis., **29**, 321.

HALL, I. C. 1922 Differentiation and identification of the sporulating anaerobes. Jour. Infect. Dis., **30**, 445.

HALL, I. C., AND RANDALL, S. B. 1922 The acid production of *Bacillus welchii*. Jour. Infect. Dis., **31**, 326.

HARRIS, J. E. G. 1919 Contributions to the biochemistry of pathogenic anaerobes. Jour. Path. and Bact., **23**, 30.

HELLER, H. H. 1920 Studies on pathogenic anaerobes. Jour. Infect. Dis., **27**, 385; 1921, Jour. Bact., **6**, 445 and 521; 1922, Jour. Bact., **7**, 1.

HENRY, H. 1917 An investigation of the cultural reactions of certain anaerobes found in wounds. Jour. Path. and Bact., **21**, 344.

HESSE, W. 1893 Ueber die gasformigen Stoffwechselproducte beim Wachsthum der Bakterien. Z. Hyg. u. Infectionskrankh., **15**, 17.

HOWE, E. C. 1912 A biometric investigation of certain non-spore-forming intestinal bacilli. Science, N. S., **35**, 225.

KENDALL, A. I., DAY, A. A., AND WALKER, A. W. 1922 Studies in bacterial metabolism. XLIV–LVI. Jour. Infect. Dis., **30**, 141.

MCINTOSH, J., AND FILDES, P. 1916 A new method for the isolation and cultivation of anaerobic micro-organisms. Lancet, **1**, 768; 1916, Compt. rend. Soc. biol., **79**, 293.

MCINTOSH, J. 1917 The classification and study of the anaerobic bacteria of war wounds. Publication of the Medical Research Committee (British), National Health Insurance. Special Report Series, No. 12.

ORCUTT, M. L., AND HOWE, P. E. 1921 Hemolytic action of a staphylococcus due to a fat-splitting enzyme. Jour. Exper. Med., **35**, 409.

REDDISH, F., AND RETTGER, L. F. 1922 *Clostridium putrificum* (*B. putrificus, Bienstock*), a distinct species. Jour. Bact., **7**, 505.

ROBERTSON, M. 1916 Notes upon certain anaerobes isolated from wounds. Jour. Path. and Bact., **20**, 327.

SMITH, T., AND SMITH, D. E. 1920 Inhibitory action of paratyphoid on the fermentation of lactose by *Bacillus coli*. I. Jour. Gen. Physiol., **3**, 21.

WEINBERG, M., AND SEGUIN, P. 1918 La Gangrene gazeuse, Paris.

WOLF, C. G. L., AND TELFER, S. V. 1917 Contributions to the biochemistry of pathogenic anaerobes. Biochemical Jour., **11**, 197.

WOLF, C. G. L., AND HARRIS, J. E. G. 1917 Contributions to the biochemistry of pathogenic anaerobes. Jour. Path. and Bact., **21**, 368 and **22**, 1; Biochemical Jour., **11**, 213.

WOLF, C. G. L. 1918 Contributions to the biochemistry of pathogenic anaerobes. Jour. Path. and Bact., **22**, 115, 270 and 289.

THE BACTERIOLOGICAL CONTENT OF MARKET MEATS[1]

C. M. BREWER

Department of Bacteriology, University of Maryland, College Park, Maryland

Received for publication November 11, 1924

Recognizing the importance which meat possesses in the diet of man; the recent popular interest shown in the demand for wholesome and pure food, and the vast strides which bacteriology has taken in the last few years; it is quite natural to surmise that a good deal of attention has been paid to bacteria in meat. Such, however, is not the case. Indeed, it is surprising to find that practically no direct work has been done on the subject. That the bacterial content of meat is of no little material interest is beyond a doubt. This is especially apparent when one considers that meat is an ideal medium for the growth and multiplication of bacteria; that the toxins and decomposition products produced in meat may be of pathogenic import; that meat is often stored for long periods of time; and that it is exposed to contamination not only at the time of slaughtering, but through the various processes of handling. It would seem, therefore, that a certain amount of work on the determination of the number of bacteria in meat would not be amiss.

It is true that this field has not been wholly neglected, but when touched upon, it has been in a more or less incidental way. Two German investigators have worked on the decomposition products of certain bacteria found in meat. Marxer (1903) stated that Hamburger steak containing 1,000,000 bacteria per gm. was on the verge of decomposition. Weinzirl and Newton (1914), however, suggested that 10,000,000 per gram would be a more proper standard. Maurel in 1911 found a diplococcus in

[1] Thesis submitted to the faculty of the graduate School in partial fulfillment of the requirements for the degree of master of science.

meat. The United States Department of Agriculture published a monograph by Charles N. McBryde, on the "Souring of Hams Due to Bacteria." LeFevre (1917) isolated liquefying and anaerobic bacteria from Hamburger steak, and Cary (1916), in sausage purchased on the Chicago market found:

	number of times
B. coli	30
Proteus vulgaris	11
Paracolon	9
B. fecalis	8
Yeast	8
Streptococcus	5
Staph. aureus	2

With the exception of these rather meager observations, there has been no other work done on this subject, so far as I know, until very recently when the Kellogg Company issued a statement that millions of colon germs are found in meats. The statement is supplemented with tables showing the number of bacteria in beefsteak, pork chops, beef liver, corned beef, Hamburger steak, and pork liver. Indeed it was the interest aroused by this statement which prompted the present bit of research.

The subject presents many interesting features, and at the outset, it was hoped that several of the more special problems might be touched upon, such as the difference in the bacteriological counts of meats under varying conditions. For instance, counts were to have been made on samples from the interior of meat, and from exposed surfaces; also meats remaining in the store for varying lengths of time were to be examined. Successive tests were to have been run on samples which had remained in the ice box for varying lengths of time. Comparisons of the bacterial content during summer and winter were subjects which would have added interest, and possible value to the main experiment. But aside from the difficulty of obtaining accurate data from the storekeepers and butchers, it was found that time was a limiting factor. It was, therefore, decided to limit the project so as to include only the number of bacteria found in ordinary meat offered for sale in the markets, without special reference to the conditions to which the meat had been previously exposed.

Even though the field was thus narrowed, it has been impossible to allot to it the amount of time that such a subject warrants.

The meat studied was purchased from local markets; and in order to obtain fairly representative samples, it was obtained from as many different sources as possible, including some nine different stores. Necessarily, those stores were confined to a rather restricted area, so that much of the meat was probably supplied by the same wholesale dealers.

The samples had been taken from all the ordinary cuts gotten from the various animals, including beef, pork, mutton, veal, as well as prepared meats, such as sausage, bologna, pudding, Frankfurters, canned meats, chipped beef, cooked ham, etc.

TECHNIQUE

As has already been stated, almost no work has been done on this subject, so there has been no definite procedure to follow. Trituration of the meat in a mortar with sand was thought of but dismissed on account of the difficulty of carrying out the operation under sterile conditions. A ball mill was tried and found to be entirely unsatisfactory. In spite of many imperfections, a meat chopper, fitted with the fine adjustment (nut-meal grinder) was finally adopted. The meat chopper was at tached to a wide-mouthed bottle so that the grinding end extended down into the mouth of the bottle and all crevices and joints were packed with absorbent cotton. The mouth of the grinder was covered with paper. The top of the bottle, the graduates used, etc., were wrapped in paper and the whole apparatus sterilized in the hot air oven at 170°C. for two hours. Later, cotton was not used, but the whole apparatus bottle, with grinder attached, was placed in a paper bag and sealed before sterilizing.

After the apparatus had been sterilized, the meat was run through the grinder into the bottle, which had been previously weighed. The meat was then weighed in the tared jar and enough sterile water added to bring the dilution to 1:100. (In many cases it was found to be more convenient to make a 1:25 dilution.) The solution was then further diluted in test tubes,

TABLE 1

CUT OF MEAT	NUMBER OF SAMPLE	PLAIN AGAR 20 DEGREES PER GRAM	PLAIN AGAR 37 DEGREES PER GRAM	GELATIN 20 DEGREES PER GRAM	LITMUS AGAR 37 DEGREES PER GRAM	AVERAGE ON PLAIN AGAR PER GRAM	PREDOMINATING TYPES*
Porterhouse steak	1		343,333	17,300,000	125,000	343,333	Colon – – – – Putrefactive – Liquefiers – Acid formers –
	2		53,333	111,500	178,000	53,333	Colon – – Putrefactive – – Liquefiers – – Molds – Acid formers –
Averages			198,333	8,705,150	151,500	198,333	
Round steak	1	1,050,000	426,000	20,000	3,440,000	738,333	Colon – – – Liquefiers – – Molds –
	2	570,000	786,000	200,000		678,333	Colon – – – Putrefactive – – – Liquefiers – – – Molds –
	3	200,000	8,000	310,000	0	104,000	Colon – – – – Putrefactive – Liquefiers –
Averages		606,666	407,111	176,666		1,720,000	

*– – – – means predominant. – – – means large number. – – means moderate number. – means few.

Hamburger steak ..	1	840,000	395,000		2,100,000	602,500	Colon – – – –
	2	1,733,333	19,500,000	3,200,000	90,900,000	10,616,666	Putrefactive – – – – Liquefiers – – – – Colon – – – Cocci – – –
	3	38,173,333	41,250,000	35,580,000	33,500,000	39,711,666	Colon – – – Putrefactive – – Molds –
	4*	233,500,000	39,680,000	161,000,000	101,500,000	136,590,000	Putrefactive – – – – Liquefiers – – – – Colon – – –
Averages.....		68,561,666	25,206,250	66,533,333	56,575,000	46,883,958	
Brisket.....	1	14,966,666	800,000	14,800,000	1,350,000	7,883,333	Putrefactive – – – – Liquefiers – – – Colon – –
	2	979,600,000	142,000,000	868,000,000	333,800,000	510,800,000	Putrefactive – – – – Colon –
Averages.....		497,283,333	71,400,000	441,400,000	167,575,000	284,381,666	
Roast beef.....	1	304,333	706,000	100,000	344,333	505,499	Colon – – – Liquefiers – – Spreaders – Molds – Subtilis –

TABLE 1—*Continued*

CUT OF MEAT	NUMBER OF SAMPLE	PLAIN AGAR 20 DEGREES PER GRAM	PLAIN AGAR 37 DEGREES PER GRAM	GELATIN 20 DEGREES PER GRAM	LITMUS AGAR 37 DEGREES PER GRAM	AVERAGE ON PLAIN AGAR PER GRAM	PREDOMINATING TYPES
Beef heart	1				593,333		Colon --- Putrefactive –
Beef liver	1	14,946,666	8,640,000	6,500,000	13,873,333	11,793,333	Cocci ---- Putrefactive --- Liquefiers --- Colon --
	2	3,960,000		3,746,666		3,960,000	
Averages		9,473,333		5,123,333		3,960,000	
Chipped beef (sealed)	1	0	0	10,000	333,666	0	Colon Mold
Chipped beef	1	100,000		0		100,000	Undetermined
Frankfurters	1	7,183,333	8,770,000	17,046,666	68,813,333	7,976,666	Colon --- Putrefactive – Liquefiers -- Molds –
	2	1,923,333	853,333	2,586,666	410,000	1,388,333	Colon ---- Cocci ---
Averages		4,553,333	4,811,666	9,816,666	34,112,666	4,682,500	

Bologna	1		325,000	458,000	6,000	325,000	Colon – – – – Putrefactive – – –
	2	30,370,000	478,000	29,581,666		15,424,000	Colon – – – – Liquefiers – – –
	3	78,266,666	593,275,000	Liquid	20,412,000,000	335,770,833	Putrefactive – – – – Colon – – – Liquefiers – – – Subtilis – –
	4	11,233,000	619,666	17,200,000	790,000	5,929,000	Colon – – – Putrefactive – – – Liquefiers – – – Spreaders – – Subtilis – –
Averages		39,958,566	148,674,250	16,746,666	6,804,263,333	94,316,377	
Potted meat	1	1,000,000	20,000			510,000	Undetermined
Liver pudding	1	40,000	61,500	643,333	1,600,000	50,750	Colon – – – – Streptococci – – – Putrefactive – Liquefiers –
	2	592,333	820,000	230,000		706,666	Colon – – – –
	3	287,900,000	29,500,000	330,000,000		158,700,000	Colon – – – Putrefactive – – – Liquefiers – – – Streptococci – – –
Averages		96,177,777	10,127,166	110,291,111		553,152,471	

TABLE 1—*Continued*

CUT OF MEAT	NUMBER OF SAMPLE	PLAIN AGAR 20 DEGREES PER GRAM	PLAIN AGAR 37 DEGREES PER GRAM	GELATIN 20 DEGREES PER GRAM	LITMUS AGAR 37 DEGREES PER GRAM	AVERAGE ON PLAIN AGAR PER GRAM	PREDOMINATING TYPES
Cooked ham	1	700,000	920,000	630,000	615,000	810,000	Putrefactive Liquefiers Colon
	2	350,000,000	260,000	623,900,000	1,766,666	176,130,000	Cocci Colon Acid formers
	3		98,600,000	Too many to count	67,000,000	98,600,000	Colon Putrefactive Liquefiers
Average		175,350,000	33,260,000	312,260,000	25,127,333	91,513,333	
Fresh sausage	1	22,860,000	1,250,000	15,000,000	3,210,000	12,055,000	Streptococci Colon Putrefactive Liquefiers Subtilis Molds
	2	610,000	75,000	300,000	20,000	342,500	Cocci Colon

Fresh sausage..	3		29,950,000	111,000,000	59,266,666	29,950,000	Colon – – – – Putrefactive – – – Liquefier – – Molds – Chromogen –
	4	9,700,000	395,000	31,616,666		5,047,500	Putrefactive – – – – Liquefiers – – – – Colon – –
	5	57,550,000	550,000	70,000,000	905,000	29,050,000	Streptococci – – – – Colon – –
Averages.....		22,680,000	6,580,000	45,596,666	15,840,416	15,289,000	
Fresh ham.....	1	0	60,000			60,000	Undetermined
	2	3,233,333	485,000	1,760,000		1,859,166	Colon – – – – Putrefactive – – Liquefiers – –
	3†	159,333	170,000	121,666	11,500	164,666	Colon – – – Putrefactive – Liquefiers – – Streptococci – – Molds – – Subtilis –
Averages.....		1,130,888	171,666	940,833		694,607	

TABLE 1—*Continued*

CUT OF MEAT	NUMBER OF SAMPLE	PLAIN AGAR 20 DEGREES PER GRAM	PLAIN AGAR 37 DEGREES PER GRAM	GELATIN 20 DEGREES PER GRAM	LITMUS AGAR 37 DEGREES PER GRAM	AVERAGE ON PLAIN AGAR PER GRAM	PREDOMINATING TYPES
Boston minced ham	1	2,583,333	650,000	2,320,000	970,000	1,616,666	Colon – – – – Putrefactive – Spreader –
Minced ham	1	403,750,000	251,100,000	338,700,000	286,000,000	327,425,000	Cocci – – – – Molds – – – Colon – – –
Smoked ham	1‡	85,000	10,000	0	90,000	47,500	Colon – – – – Acid formers – –
Smoked shoulder.....	1‡	20,000	300,000	30,000	0	160,000	Colon
Smoked bacon.....	1‡	1,120,000	1,287,333	458,333		1,205,666	Colon – – – Putrefactive –
	2		10,000	0		10,000	
Averages.....			648,666			607,833	
Pork chop	1	2,866,666	2,400,000	3,373,333	1,426,666	2,633,333	Colon – – – – – Putrefactive – – – Liquefiers – – – Spreaders – Acid formers –

Pork chop	2	5,566,666	3,133,333	2,533,333	3,500,000	4,349,999	Colon – – – – Cocci – – – Streptococci – Chromogen – Subtilis –
	3	32,706,666	6,223,333		5,800,000	19,464,999	Colon – – – Putrefactive – – –
Averages		13,713,333	3,919,888	2,933,333	3,575,555	8,816,666	
Lamb chop	1	10,000	10,000			0	Colon
	2	3,916,666	4,033,333	5,400,000	6,400,000	3,974,999	Putrefactive – – – – Liquefiers – – – Colon – – Spreader – Chromogen –
	3	145,500,000	15,906,666			80,703,333	Colon – – – Putrefactive – – –
	4	1,150,000	1,146,666	480,000	520,000	1,148,333	Colon – – – – Putrefactive – – Liquefiers – –
Averages		37,644,166	4,274,166	2,940,000	3,485,000	20,959,166	

TABLE 1—*Concluded*

CUT OF MEAT	NUMBER OF SAMPLE	PLAIN AGAR 20 DEGREES PER GRAM	PLAIN AGAR 37 DEGREES PER GRAM	GELATIN 20 DEGREES PER GRAM	LITMUS AGAR 37 DEGREES PER GRAM	AVERAGE ON PLAIN AGAR PER GRAM	PREDOMINATING TYPES
Lamb kidney	1	1,105,000	100,000	780,000	940,000	602,500	Colon Putrefactive Liquefiers
	2	0	30,000	0		30,000	Chromogen
Averages		525,500	65,000	390,000		316,250	
Veal	1		310,333	481,000	1,000	310,333	Colon Putrefactive Liquefiers
	2	65,240,000		8,693,333	37,133,333	65,240,000	Colon Putrefactive Liquefiers Spreaders Molds
	3	3,125,000,000	1,984,000,000	Liquid	142,973,333	2,554,500,000	Colon Putrefactive Liquefiers Acid formers
Averages		1,595,120,000			60,335,888	873,350,111	

Veal kidney.....	1	165,000	376,666	4,133,333	10,000	270,833	Colon	— — — —
							Putrefactive	— —
							Liquefiers	— —
Veal chop.....	1	1,600,000	505,000	933,333	600,000	1,052,500	Putrefactive	— — — —
							Liquefiers	— —
							Colon	— —
							Spreaders	— —
							Chromogen	— —

*In ice-box forty-eight hours.
† In ice-box thirty-six hours.
‡ In ice-box twenty-four hours.

by means of sterilized water blanks. Dilutions of 1:10,000; 1:100,000, and 1:1,000,000 were made and plated on litmus agar at 37°C., plain agar at 37°C., plain agar at 20°C., and gelatin at 20°C. The plates were incubated for three to four days before counting.

Inasmuch as specific identification of all the bacteria found in the meat would have consumed an undue amount of time and labor, it was thought best to run semi-qualitative tests on the samples. Therefore, an effort was made only to determine the general types of bacteria which infect market meat. In this connection, the samples were plated on litmus agar for the purpose of determining acid-formers. Gelatin was used to designate the liquefiers. Molds, chromogens, the colon group, putrefactive organisms (to a lesser degree), streptococci, *B. subtilis*, *B. mycoides* etc., were readily distinguishable from the appearance of the colonies. Carbol fuchsin stains were made of suspected cocci, and of any unusual colonies.

The majority of the samples of meat were ground and plated within one-half to one hour after they reached the laboratory, or about two hours after being purchased. In the rest of the cases, the meat was kept in an ice box not longer than five hours before "running it through."

As has been intimated, the procedure at best admits of considerable error. In using the finest grinder obtainable, the meat was ground so that varying portions of it were small enough to remain in suspension, but the remainder was left in comparatively large particles, which settled out, and were capable of harboring thousands of bacteria. After the experiment had been progressing for some time, the test of shaking the solution in a machine was tried. It was found that the results, when compared with those of samples shaken by hand, varied slightly but not appreciably. And considering the added risk of contamination during the transference to the shaking bottles, mechanical shaking was regarded as being of insufficient value to offset the disadvantages involved. Another weakness manifest in the technique was the diluting of the samples. During this operation, the meat was liable to contamination.

The work necessarily proceeded but slowly, partly due to lack of sufficient apparatus. Sterilization in the autoclave proved to be unsatisfactory on account of the large amounts of water which condensed in the jar, with a consequent inaccuracy of weighing. Furthermore, the thick glass jars would not stand the sudden rise in temperature. For this and minor reasons there was insufficient time to run more than one or two samples on many of the different cuts of meat, whereas five or six samples would have been of value in establishing a truer average.

TABLE 2

General summary

TYPE OF MEAT	NUMBER OF SAMPLES	NUMBER OF BACTERIA PER CUBIC CENTIMETER
Fresh beef	15	48,147,097
Fresh pork	6	4,765,636
Smoked pork	4	363,944
Fresh lamb	6	10,637,708
Fresh veal	5	291,557,815
Compositions or prepared meats*	19	83,999,335

* Excepting smoked or cured meats.

DISCUSSION

An interesting feature of the results, as can be seen from the data in table 1, was the wide difference in the number of colonies which often existed between the plates incubated at 20°C. and those incubated at 37°C. In many cases not only did the number of colonies differ on the two sets of plates, but the nature of the colonies presented a marked contrast in appearance. Very often the resemblance between the agar plates at 20°C. and the gelatin plates, incubated at the same temperature, was more striking than was the resemblance of the two sets of agar plates, incubated at different temperatures. It was usual for the number of colonies grown at 20°C. to far exceed those grown at 37°C., though in a few cases those on the 37° plates equalled or outnumbered the former. The average number of colonies per gram, on agar, at 20°C. was 123,814,009 and at 37°C. was 65,200,708. A partial explanation of this phenomenon lies in the

fact that practically all the meat was purchased during cool weather. It was keenly regretted that it was impossible to continue the experiment through the summer months, in order to note the influence warm weather would have on this phase of the subject.

In viewing the tables, it should be noted that the colon group is a very persistent invader in all types of meat, in fact, its colonies far outnumbered all others. Putrefactive organisms were easily second. Pathogenic organisms occur rarely in market meats, no doubt due largely to the rigid Government inspection. Local contamination in the market, after the meat has passed inspection, would account for most of the pathogens found. In this experiment large numbers of cocci, including short-chained streptococci, were observed, but in such cases the organisms grew much more abundantly, or solely, on the 20°C. plates.

Unfortunately, the table giving the general averages for the numbers of bacteria in the different types of meat may be somewhat misleading. For instance, the data show that veal contains a much higher average than all the other meats, whereas it is probable that the prepared meats were really higher in count than other meats. The probable deception in this case is undoubtedly due to the small number of samples used in calculating the veal average, one high count sample raising this average much higher than did high counts in cases where a larger number of samples were examined. The figures given for smoked meats, however, are more uniform. It will also be seen that the bacterial content is much lower than in the case of fresh meats. This is no more than would be expected as the water content of smoked meats is lowered, thus affording a less favorable medium for the growth of bacteria. Also, many bacteria are doubtless killed at the time of smoking and curing.

It is very difficult, if not impossible (at least for the untrained eye), to ascertain the comparative contaminations of moderately fresh meat. Where many of the high counts were obtained, no clue was given by the appearance of the meat. As a rule, of course, meat which was not in good condition when purchased,

contained great numbers of bacteria. But this was not necessarily true as in one or two instances, meat which did not appear to be particularly fresh gave only a moderate number of colonies when plated.

Little work has been done on determining at what stage, during the process which the meat undergoes in reaching the consumer, the bulk of contamination enters. Conradi and others have found bacteria in muscles of healthy animals. He expressed the opinion that they were absorbed from the intestines along with the food. It is reasonable to suppose, however, that much of the infection occurs at the time of slaughtering, while additional bacteria are acquired during each successive handling.

It may be well to note that these data have been obtained from a comparatively few determinations. The samples were purchased from small meat markets. The counts may, therefore, be somewhat higher than those obtained from the large markets, for in them the meat is necessarily handled more efficiently.

To offset this condition, however, no attempt was made to cultivate or count the anaerobic bacteria which were present in the meat. The sub-surface colonies which formed in the agar plate were facultative anaerobes, and largely organisms of decay; but the true putrefactive bacteria being anaerobes will not grow in the presence of atmospheric oxygen.

CONCLUSIONS

The conclusions which may be drawn are as follows:

1. Counts from different samples of the same cuts of meat usually varied more than those from different meats.
2. Prepared meats usually contained more bacteria than fresh meats.
3. Smoked meats contained a much smaller number of bacteria than fresh meats.
4. Of the bacteria encountered in the different meats, the colon group predominated.
5. The appearance of fresh meat could not be taken as an index to its bacterial content.

ACKNOWLEDGMENT

I wish to acknowledge my indebtedness and express my thanks to Dr. E. M. Pickens of the Department of Bacteriology for assistance in outlining and supervising this work.

REFERENCES

CARY, W. E. 1916 The bacterial examination of sausages and its sanitary significance. Amer. Jour. Pub. Health, **6**, 124–135.

LE FEVRE, E. 1917. The manufacture of meat food products. Amer. Food Jour., **12**, 140–142.

MARXER, ANTON 1903 Beitrag zur Frage des Bakteriengehalts und der Haltbarkeit des Fleisches. Strassburg, Els.-lathr.druckerie.

MAUREL, E. 1911 The occurrence of microörganisms in the interior of meat, potatoes, and sausage. Compt. Rend. Soc. Biol., **70**, 241–244; Exp. Sta. Rec., **25**, 265.

MCBRYDE, C. N. 1907 A study of the methods of canning meats with reference to the proper disposal of defective cans. Twenty-fourth Ann. Rep. Bureau of Animal Industry, U. S. Dept. Agr., 279–296.

WEINZIRL, J., AND NEWTON, E. B. 1914 Bacteriological analysis of hamburger steak with reference to sanitary conditions. Amer. Jour. Pub. Health, **4**, 413–417.

LONG TUBE METHOD OF CULTIVATING MICRO-ORGANISMS WITH OBSERVATIONS ON MOBILE COLONIES IN LIQUID MEDIUM

LEWIS B. BIBB

Marine Corps, United States Army

Received for publication December 5, 1924

The automatic transferring apparatus devised by Felton and Daugherty (1924) suggested to the author the idea of attempting to induce continuous growth of microörganisms through ordinary media contained in glass tubes several feet or yards in length. This plan appeared commendable because it would obviate the mechanical and other disturbance incident to transfer, and would afford a continuous view of the organisms reacting to their environment. It was found that the progress of the organisms through the tube could easily be followed and the degree of activity thus estimated quantitatively.

A stock strain of *Escherichia coli* (*B. coli*) obtained at the Army Medical School and known as "S3" was used in the earlier experiments. Later an organism belonging to the colon group was isolated from feces and grown as described. This organism will be referred to as "S4."

TECHNIQUE

Ordinary glass tubing of 3 to 5 mm. outside diameter was autoclaved in 5-foot lengths and these lengths sealed end to end in the blast lamp until a tube 30 feet long was obtained. Under aseptic precautions, meat extract broth of pH 7.6 was aspirated into the tube, care being taken to avoid air bubbles. One end of the tube was then sealed in the blast lamp, and the open end was cut off 1 inch longer than the column of contained broth. Inoculation was done by introducing about 1 c. mm. of a twenty-four-hour slope culture of one or other of the organisms referred to

above. With a wire loop, the inoculum was emulsified in the broth near the open end of the tube. The tube was then left in a horizontal position at room temperature (76° to 89°F.).

For purposes of description, the inoculated end of the tube will be called the rear or proximal end; the other end, the forward or distal end. The most distal or forward visible growth will be referred to as the "head of the column" of organisms.

It was found by repeated trials that the progress of the bacilli from proximal to distal end of the tube could be satisfactorily followed with the unaided eye, as will be seen from the following: After dividing the tube into two at a point $\frac{1}{4}$ inch in advance of the head of the column, it was often found that growth later occurred in the distal portion of the tube. When, however, the tube was divided at a point $\frac{1}{2}$ inch in advance of the head of the column, the distal segment of the tube remained sterile, thus indicating that visible turbidity kept up to within $\frac{1}{2}$ inch of the most advanced bacilli. That is, clearness of medium could be reliably accepted as proof of sterility except as regards the $\frac{1}{2}$ inch of medium immediately in advance of recognizable turbidity.

Consequently, when turbidity was accepted as a guide to the progress of the bacilli from proximal to distal portions of the tube, the limit of error was within $\frac{1}{2}$ inch. In the experiments reported in the following paragraphs, the rate of progress of the organisms through the tube was always judged by observations of turbidity.

The S3 organism grew and progressed for twelve days without change in speed or other visible alteration. At the end of this time it had reached the end of the tube, and the experiment was terminated. The rate of progress was smooth and uniform, and in a dozen tubes averaged about 4 feet per day or 5 cm. per hour. The S4 organism progressed at a uniform but slower rate.

The turbidity regularly took on a characteristic form. It was more opaque at the head of the column. The turbidity produced by the S3 organism when viewed in profile, appeared as one or two triangular areas of dense whitish turbidity separated by a streak of relatively clear medium 1 mm. wide and running transversely across the tube. About 3 to 5 cm. in rear of the head of the column appeared a second triangular turbidity, similar

to the first, and this was also in some instances bifid or double. Between these two densely turbid areas was a segment of almost clear medium 3 to 5 cm. long. The S4 organism produced a thin elliptical disc of turbidity inclined at an angle of about 45 degrees and sloping from below upwards and backwards, as can be seen in figures 1 and 2. This peculiar formation constitutes the head of the column in S4 cultures. It was usually $\frac{1}{4}$ to $1\frac{1}{4}$

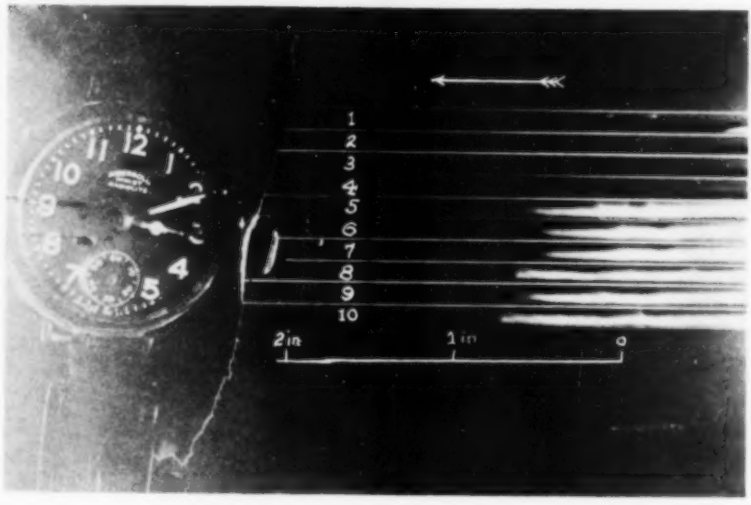


FIG. 1. BROTH TUBE CULTURES OF ORGANISM S4, COLON GROUP

Tubes 1, 2, 3 and 4 contain cultures three hours old. Tubes 5, 6, 7, 8, 9 and 10 contain cultures six hours old. Inoculated end of tubes on right. Organisms moving towards the left. One mobile colony was visible in each of tubes from 5 to 10 inclusive. The mobile colony is about $\frac{1}{4}$ inch to the left of the main mass of turbidity. It is disk-shaped and inclined at an angle of about 45 degrees.

inches in advance of the main mass of turbidity. In rear of the turbid disc, followed a segment of almost clear fluid medium, and then in turn followed the main mass of turbidity as shown in figures 1 and 2. If the tube was twirled until the disc became hazy, it soon formed up again, took on its charaeteristic shape and angle, and proceeded as before.

The peculiarities of outline of the main turbidity persisted as the mass progressed through the tube. In figures 1 and 2, a sharp peak of turbidity can be seen about the middle of the main mass of turbidity in tubes 8 and 10. During the fifteen-minute intervals intervening between the two photographic exposures, this peak had progressed about $\frac{1}{4}$ inch.

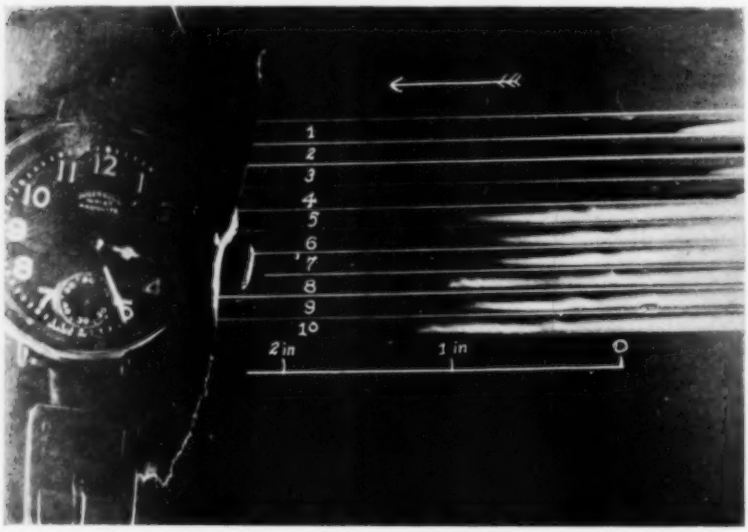


FIG. 2. SAME TUBES SHOWN IN FIGURE 1, BUT FIFTEEN MINUTES LATER

Comparison of figures 1 and 2 will show that practically all landmarks persisted, although they moved to the left nearly $\frac{1}{4}$ inch during the fifteen-minute interval.

In figure 1, dark crescentic areas indicating lack of turbidity are seen about the middle of the main turbidity in tubes 5, 6 and 7. In figure 2, these areas are seen to have progressed a certain distance through the tube, while maintaining their individuality. Some alteration in the arrangement of the head of the main mass of turbidity is seen in tube 8.

Tubes 1, 2, 3, and 4 illustrate the progress of turbidity during the first three hours after inoculation. During this early period,

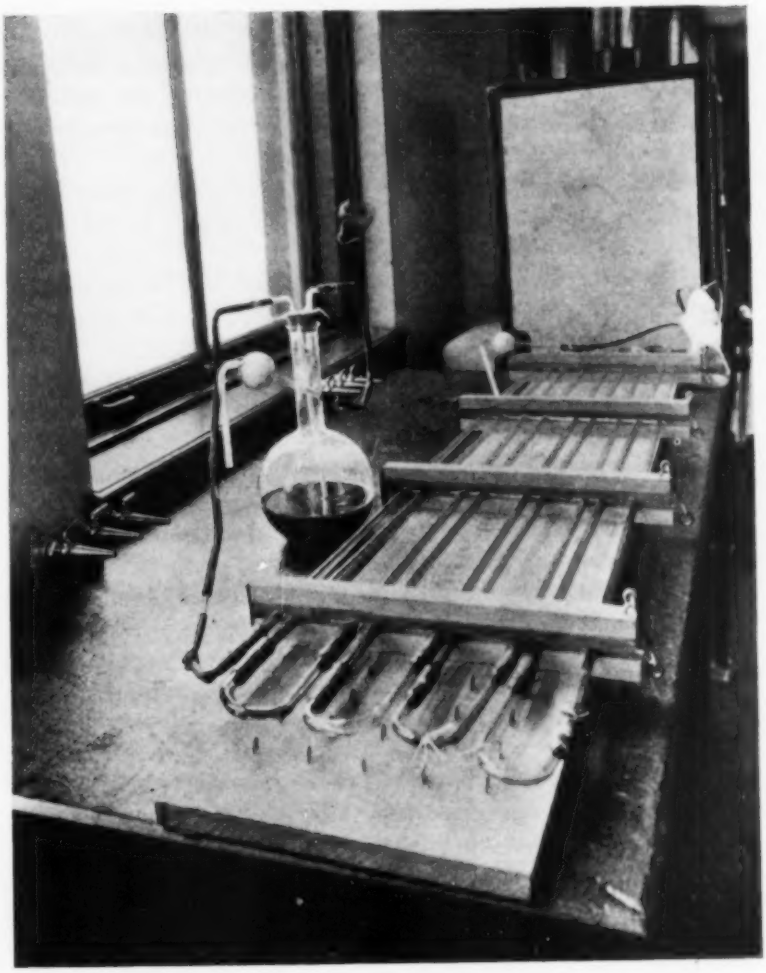


FIG. 3. TUBE 50 FEET LONG AND 1 CM. IN DIAMETER, LEVELLED FOR AEROBIC CULTURE

Joined with rubber cuffs, collodionized at joints, placed in frame, and entire apparatus including two flasks of broth autoclaved "en bloc."

the bacteria appear to be, as it were, walking on the bottom of the tube.

The mobile colonies of S3 also, when twirled, lost character and became a vague haze, but after five minutes the turbidity again took on its usual outline. This process was repeated six times with the same result.

After the second day, the turbidity in the rearmost or proximal portions of the tube settled and formed a whitish streak about 1 mm. wide in the bottom of the tube.

In one experiment, S3 was grown under aerobic conditions. A larger tube was used, 1 cm. in diameter. It was levelled carefully, and fluid medium was run in until it stood at the desired level. This was done only after repeated trials. Figure 3 shows such an aerobic tube 50 feet long. The organism grew through this tube at an average rate of 3 linear feet per day.

DISCUSSION

This method of following the progress and estimating the activity of microörganisms is economical of time and materials. The method is favorable for observing the effect of different substances or influences on bacterial activity.

It appears that the bacilli investigated practically never diffuse uniformly through liquid media contained in glass tubes in horizontal position. Several explanations of their behavior suggest themselves. The colonies could be conceived to be due to more favorable conditions for growth at certain focal points. If the favorable conditions were due to substances dissolved in the fluid, it is difficult to understand how the mobile colony could carry them along with itself. This is evidently done, however, for the successive colonies in many instances pass any given point like a procession. It appears likely that the favorable conditions either inhere or are attached to the bacilli themselves, and that they act as a group, carrying along with them their favorable conditions as they progress. As individuals, they seem to manifest positive tropism towards each other. As a group, the mobile colony appears to manifest positive tropism towards the adjacent sterile broth.

The persistence of form manifested by the advanced disc in S4 cultures and the advanced triangle in S3 cultures suggests that the bacteria forming these are entangled by their cilia, and thus constitute mobile colonies in liquid medium.

For the S3 culture, and for assistance and counsel, thanks are due Major Henry J. Nichols, M.C., Army Medical School. For the S4 culture thanks are due Captain Charles B. Spruit, M.C., U. S. Army. For the photographs I am indebted to the enlisted photographers, Walter Reed General Hospital, and to Private first class George Bowers, Medical Department, U. S. Army.

SUMMARY

1. Bacilli of the colon group have been cultivated in glass tubes 30 feet long and 3 mm. in diameter.

2. These bacilli progress through the tube at a uniform rate, usually about 4 feet per day for S3 and about 2 feet per day for S4.

3. Instead of diffusing uniformly through the tube, the bacilli form one or more relatively compact turbid areas called mobile colonies.

4. Bacilli of certain strains regularly form mobile colonies of characteristic shape, and if such colonies are disturbed by twirling of the tube, they again form themselves into the usual shape after a few minutes.

REFERENCE

FELTON AND DAUGHERTY 1924 Jour. Exper. Med., **39**, 137, 155.

CLUB-FORMATION BY ACTINOMYCES HOMINIS IN GLUCOSE BROTH, WITH A NOTE ON B. ACTINOMYCETUM-COMITANS

STANHOPE BAYNE-JONES

Department of Bacteriology, School of Medicine and Dentistry, University of Rochester, Rochester, New York

Received for publication December 18, 1924

The mode of formation and function of the club-shaped structures found in the granules in the pus from lesions due to *Actinomyces* have interested bacteriologists since the first observations upon the "ray fungi." During this period two theories have been advanced to account for these "clubs"; one attributing to them a developmental part in the life cycle of the organism, the other regarding them as thickenings of the sheaths enclosing the filaments as a protection against the effect of animal fluids. Most of the writers on the subject have accepted the latter view, especially since the work of Wright (1905), who, although able to obtain growths of *Actinomyces bovis* and *Actinomyces hominis* exhibiting "clubs," succeeded in bringing about the formation of clubs only in media containing serum or similar animal fluids.

The observations to be recorded in this paper indicate that it is not correct to regard club-formation by *Actinomyces* as exclusively the result of an interaction between the organism and animal fluids. As will be shown, club-formation may take place in simple media, free from serum and other animal protein. This report will include observations on the manner of growth of a filament from a club, and a note on a culture of *B. actinomycetum-comitans,* which apparently has not been hitherto described in this country.

The material from which the strain of *Actinomyces hominis* was isolated was pus obtained by aspiration of a cervical lymph node of a patient, Yurick, at the Johns Hopkins Hospital in 1920.

The bacteriological studies were made in the laboratory of the Department of Pathology of the Johns Hopkins Medical School.

As the material was obtained from a closed lesion, the troublesome secondarily invading bacteria were fortunately absent. The pus contained typical "sulphur granules," composed of leucocytes and clusters of the branched filaments and clubs characteristic of the ray fungus. In an unusual Gram stain, the branched filament retained the purple dye, while the sheath and its club-like thickening stained brilliantly with saffranin. The appearance of this preparation is indicated in the microphotograph reproduced as figure 1, plate 1. There were Gram-negative particles among the filaments in the pus, but these were at first regarded as detritus.

The cultures were obtained by placing the pus containing the actinomycetic granules in tubes of 1 per cent glucose meat infusion agar and 1 per cent glucose meat infusion broth. The media had been sterilized in the autoclave and neither serum nor ascitic fluid was added. Their reaction was pH 7.6. The pus was distributed by means of a glass pipette throughout the length of the column of agar, about 10 cm., in the tube containing it, after the agar had been melted and cooled to 40°C.

After five days' incubation at 37°C. there was definite growth in both sets of tubes. In the tubes containing the glucose agar, the growth formed a granular yellowish ring about 2 mm. in thickness at a level about 1 cm. below the surface of the agar. The organism was not a strict anaerobe, but grew slightly on the surface and fairly abundantly under reduced oxygen tension. It was characteristically a microaerophile. In the bottom of tubes of glucose broth, yellowish chalky granules gradually increased in size until some reached a diameter of 1 mm. or more, having irregular shapes. These granules and the colonies in the agar were composed of tangled filaments, which were Gram-positive. The filaments branched and showed a great deal of granular fragmentation. They were easily broken up into bacillary forms by crushing the colony on a slide. In the ordinary preparations, made by smearing the granules on slides, no distinct sheath or club-formation could be seen.

In order to preserve the natural arrangement of the filaments and their clubs, the colonies obtained from the second and third generations of the growth in glucose broth were fixed, embedded in paraffin, sectioned and stained. The fixing fluid used was Bouin's, prepared as follows:

	number of parts
Picric acid (saturated aqueous solution)	75
Formol (40 per cent)	25
Acetic acid (glacial)	5

The broth was decanted from the tube containing the sediment of the growth of *Actinomyces* and 10 cc. of Bouin's fluid was poured in over the colonies. After twenty-four hours, the colonies were lifted out gently by means of a large caliber pipette and dropped into water in order to wash them. By similar manipulations, they were dehydrated in alcohol and embedded in paraffin in the usual manner. The sections finally obtained were fixed to glass slides and stained by a modified Gram method, using carbol methyl violet B in place of aniline gentian violet, and counterstaining with aqueous eosin. At the end, they were passed rapidly through absolute alcohol or blotted dry, and mounted in balsam under a cover slip.

In section, the colonies had the same appearance as those shown in the illustrations accompanying Wright's (1905) paper. They were hollow or curved like part of a shell. Near the outer edge there was a densely staining convex mass of tangled filaments. It seemed as if this region had been the most active zone of growth and that on account of the unilateral extension of the growth in this part of the colony a curvature of the mass had been produced. The appearance of a section of a colony is shown in figure 9, plate 1.

The filaments at the inner and outer edges of this zone contained a central core of Gram-positive substance enclosed in an eosin staining sheath of hyaline material. Many of the freely projecting filaments closely resembled the clubs seen in the granules from the lesion of the patient with actinomycosis, and looked exactly like the structures found by Wright in sections of the colonies in his cultures. These formations are shown in the microphotographs, figures 2 to 8, plate 1.

The relation of the sheath to the club-shaped extremity appeared most clearly in a detached branching filament. The sheath could be seen covering the whole length of this granular filament, gradually thickening to a moderately bulbous extremity, forming a club at the end of the filament (fig. 2, plate 1).

Numerous attempts were made to determine what portion of the club took part in the growth of the organism. To do this, the granules in the original pus were mashed between sterile slides, emulsified in drops of salt solution, and transferred to the cover glass of the moist chamber used in micropipette manipulations. By means of a Chamber's pipette holder, single clubs were picked out of these drops, transferred to drops of glucose broth on sterile cover slips and incubated as hanging drops over hollow ground slides. Most of the clubs thus isolated failed to show any change. In two instances, however, growth of the filament took place slowly, reaching a length of about 50 to 100 micra in ten days, and then stopped. This growth occurred at the thin tapering proximal end of the club, as shown in the accompanying drawings (text-fig. 1). There was no change in the club itself. The proliferative part of the structure seemed to be the remnant of thin filament attached to its inner narrow base. This suggests that the thickened sheath composing the club is an older, swollen part of the same sheath which covers the filament, while the actively growing region of the organism is a lower part of the filamentous stem or mycelium, which by lengthening, pushes the club forward.

B. ACTINOMYCETUM-COMITANS

It has been mentioned that among the filaments of *Actinomyces* in the pus from the lesion there was in addition to leucocytes a granular Gram-negative material. These granules were not thought to be bacteria until the cultures were examined. In the smears from the colonies of *Actinomyces* in glucose agar and broth, this Gram-negative granular material reappeared in abundance, and in the glucose broth cultures there were flakes adhering to the sides of the tubes which were found on examination to be composed of small Gram-negative cocco-bacillary

forms. When these flakes were transferred to fresh tubes of glucose broth they slowly increased in size and number, some sinking to the bottom, others adhering to the sides of the tubes. On the surface of glucose agar, the growth from one of these flakes formed a thin, dry, granular slightly yellow, hard growth, which clung to the medium. Acid without gas was formed in glucose and lactose broth. Gelatin was not liquefied. The

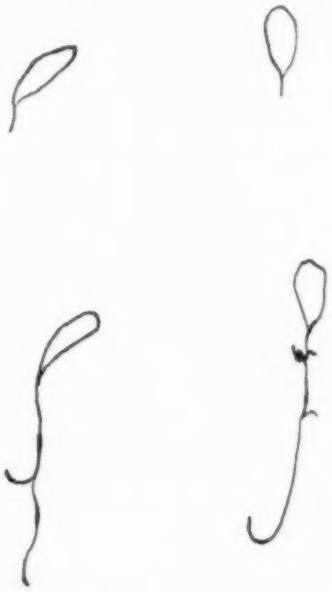


FIG. 1. GROWTH OF FILAMENTS OF ACTINOMYCES HOMINIS FROM THE PROXIMAL ENDS OF THE CLUBS

Drawings of isolated clubs in hanging drop cultures. Top row; original appearance of the club. Bottom row; same club after ten days incubation, showing the growth of branched filaments from them.

organism was not motile. In subsequent transfers, in a new batch of glucose broth, the growth produced turbidity as well as yellowish flakes.

Morphologically and culturally, this cocco-bacillus seemed to correspond to an organism first described by Klinger (1912) and

named by him *B. actinomycetum-comitants.* This trinomial will be changed ultimately to conform with the standards of bacteriological nomenclature, but it does not seem worth while to do that until we know more about the organism. Colebrook (1920) found the same organism in 80 per cent of the cases of human actinomycosis studied by him, and as in the case recorded in this paper, obtained the organism from the pus of a closed lesion. Its presence in a closed lesion seemed to exclude the possibility of its entrance into the lesion from the immediate exterior. This observation is the first report of the occurrence of *B. actinomycetum-comitans* in this country, but does not advance the knowledge of its significance beyond the facts noted by Colebrook (1920), to whose paper reference is made for a complete statement of all that seems to be known about this bacterium. Colebrook, who has compared the cultures of *B. actinomycetum-comitans* isolated by him with a culture of the "actinobacillus" of Lignières and Spitz is of the opinion that the two organisms are different. *B. actinomycetum-comitans* does not grow conspicuously except when alone in a tube of medium. It can be easily overlooked, being mistaken for detritus in the pus of lesions or for degenerated particles of *Actinomyces* in culture media. It is suggested that it be searched for particularly by bacteriologists dealing with actinomycosis, in order to accumulate the data needed to explain its presence and significance in the lesions and to establish its position among the bacteria.

SUMMARY AND CONCLUSIONS

Cultures of *Actinomyces hominis*, isolated from a closed lesion of a patient with actinomycosis, were grown in glucose agar and glucose broth without the addition of serum or any other animal protein. The colonies formed in the glucose broth were fixed in Bouin's fluid, embedded in paraffin, sectioned and stained by a modified Gram's method. The natural arrangement of the filaments was preserved in these sections and club formation could be studied advantageously. The filaments at the edges of the colonies were found to be enclosed in sheaths of hyaline material which terminated in bulbous thickenings over the ends of the

filaments. This type of club formation closely resembled the clubs seen in granules of the ray fungus in pus, and demonstrated that the characteristic clubs can be formed by organisms growing in simple media. It is concluded, therefore, that animal fluids are not essential as a stimulus to club formation by *Actinomyces hominis*.

When isolated clubs from the original material were observed in hanging drops of glucose broth, growth in two instances was seen to take place from the portion of the filament attached to the proximal narrow end of the club. The bulbous portion of the club took no part in growth in these cultures.

B. actinomycetum-comitans (Klinger, 1912) was found associated with the strain of *Actinomycetes hominis* isolated from this patient. This is the first recorded observation of the occurrence of this organism in the lesions of actinomycosis in this country.

REFERENCES

COLEBROOK, L. 1920 The mycelial and other microörganisms associated with human actinomycosis. Brit. Jour. Exper. Path., **1**, 197–212.

KLINGER, R. 1912 Untersuchungen über menschliche Aktinomykose. Centralblt. f. Bakt., etc., I Abt., Orig., **62**, 191.

WRIGHT, J. H. 1905 The biology of the microörganism of actinomycosis. Jour. Med. Res., **13**, 349–398.

EXPLANATION OF PLATE 1

FIG. 1. Microphotograph of a preparation of branching clubs of *Actinomyces hominis*, showing the appearance of these structures in the original pus from the lesion. Gram stain. Gram-positive central filaments surrounded by hyaline Gram-negative sheaths. × 3000.

FIG. 2. Isolated branched filament from a culture of this strain of *Actinomyces hominis* in glucose broth. This shows the relation of the sheath covering the granular filament to the bulbous thickening composing the club. Photograph of Gram-stain preparation. × 3000.

FIG. 3, 4, 5, 6, 7, 8. Clubs and filaments enclosed in sheaths formed at the edges of colonies of the culture of *Actinomyces hominis* in glucose broth. Photographs of sections stained by Gram stain. × 6000.

FIG. 9. Cross section of a colony of *Actinomyces hominis* from the growth in glucose broth. This shows the curvature and hollowness of the colony and the thick zone of proliferation near the outer edge. Photograph of Gram stain preparation. × 200.

(Bayne-Jones: Club formation by actinomyces hominis.)

STUDIES IN MICROBIC HEREDITY

II. THE SEXUAL CYCLE OF B. COLI IN RELATION TO THE ORIGIN OF VARIANTS WITH SPECIAL REFERENCE TO NEISSER AND MASSINI'S B. COLI-MUTABILE

RALPH R. MELLON

From the Department of Laboratories, Highland Hospital, Rochester, New York

Received for publication August 7, 1925

INTRODUCTION

Perhaps few questions in bacteriology have aroused more discussion, often bitter in character, than has that of bacterial mutation. Likewise, on few questions has there prevailed more ignorance of the fundamental biology that of necessity underlies variability of any degree, be it great or small. Accordingly, the great volume of literature that has grown up about the subject has consisted almost wholly of observations that have lacked the common denominator of a guiding principle.

The first paper of this series (1925), predicating as it does a sexual cycle for the bacteria, makes possible a theory or mechanism for this important phenomenon which is grounded in botanical considerations. The reader is referred to this paper for morphologic detail and other supporting evidence which is at the same time so intimately related to the material to be presented here. This theory has been applied to the developmental mechanism involved in the genetics of that classical variant of the enteric organisms, namely, the *B. coli-mutabile* of Neisser (1906) and Massini (1907). Inasmuch as their work has been so frequently confirmed, and particularly by the single cell method, its relation to our theory in respect to the mechanism involved becomes of signal interest.

Irrespective of the mechanism of variation the validity and frequency of the phenomenon has become more firmly established

as a result of the influence of the bacteriophage, an agent whose action in this respect is subject to rather definite control. Although the studies of d'Herelle (1922) with the bacteriophage have disclosed no botanical mechanism for his variants, it is pertinent that he invokes the sexual hypothesis as the most probable explanation for the origin of certain of his resistant coccoid forms, which recall our "giant coccoids"—more precisely, zygospores and gonidia.

The work begun by Rodet (1894) on the pleomorphism of *B. coli* and followed by that of Adami and Abbott (1899) on a coccus phase for this same organism pointed the way for a host of similar studies which leave no doubt of the great lability of the group of enteric organisms. In addition to the striking work of Neisser and Massini (loc. cit.) with *B. coli-mutabile* we have also the work of Penfold (1911) and others who associate variation with secondary colony formation, without however, apprehending the botanical nature of this formation. The work of Prell (1917) on the cyclomorphism of *B. coli* and of Eisenberg (1918) and of Bergstrand (1923, 1924) each deals with a different aspect of the problem, while those of Arkwright (1921), Andrewes (1922) and Breinl (1922) have a singular practical significance in diagnostic work involving the agglutination-adsorption reaction.

To appreciate the desirability for some fundamental generalization that will help correlate the mass of disconnected observations with modern genetic theory one need read but one paper in this field—that of Prell (1917)—and attempt to decipher his fluctuations, modifications, transformations and variations!

EXPERIMENTAL

On the genetic origin of Neisser and Massini's B. coli-mutabile

These authors describe a strain of *B. coli* isolated from a case of enteritis that failed to ferment lactose when planted on an Endo plate except under the following conditions: When the plates were left at 37° for from 72 to 96 hours "red" or lactose-fermenting daughter colonies appeared in the "white" or non-lactose-fermenting ones. On transfer to a fresh Endo plate the "red" colonies promptly form acid and breed true, while the

"white" margins of the mixed colonies or the all "white" colonies yield non-lactose-fermenting colonies unless they be left at 37° until the daughter or secondary "red" colonies develop. No daughter colonies are formed from the "red" colonies. Although their cultures were not started from single cells as were those of Kowalenko (1910) and others who confirmed their work, the careful nature of their plating experiments leaves little doubt of the correctness of the observations of these authors. Their experiments do not reveal why all the bacilli of the "white" colony do not become "red" nor indeed why any of them should do so; neither do they intimate how such a peculiar strain of *B. coli* came into being, all of which points our experiments attempt to explain.

The *B. coli* studied by us was the Nx strain described in the first paper of this series (Mellon, 1925). As indicated there its capacity to develop conjugating forms from large fungoid filaments was conspicuous. A single celled culture of this strain was planted in glycerophosphate broth of a pH of 6.8 at 37°C. This broth was prepared by adding 1 per cent disodium glycerophosphate to a veal infusion containing 1 per cent pepton and 0.5 per cent NaCl, as suggested by us in a previous paper on the subject (1921). After twenty-four to forty-eight hours in this broth the culture showed a pellicle containing myriads of the pleomorphic elements just mentioned.

The culture after standing two months at room temperature was plated out on a glycerophosphate Endo agar, and there were dissociated from the rather rough spreading colonies of the normal form of the organism a few smooth circumscribed small colonies which were very mucoid in character. In contrast to the normal form the Endo medium was unaffected by these colonies, which as we will show were the immediate ancestors of the *B. coli-mutabile*. When transferred to an agar slant for twenty-four hours these colonies grow as large coccoidal forms in the condensation water. Since *none of the small normal sized B. coli were present* it seems quite clear that these mucoid colonies represented a reproduction of the large coccoid forms (fig. 1) whose immediate antecedents were the zygospores developed in from twenty-four

to forty-eight hours in the broth culture. The long residence in the broth stabilized these coccoid forms so that on transplant they reproduced as such under suitable conditions. However, on the agar slant they sprouted out into coarse filaments and rods (fig. 2), which in turn showed zygospore formation in all stages of development. For example, figure 3 (at *a*) shows one of

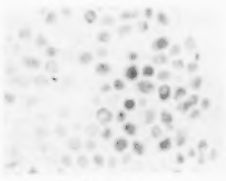


FIG. 1

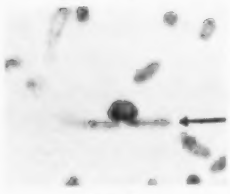


FIG. 3

FIG. 2.

FIG. 1. THE LARGE COCCOIDAL FORM OF B. COLI ORIGINATING FROM THE ZYGOSPORES; FROM A TWENTY-FOUR-HOUR ENDO PLATE; × 1200; CARBOL THIONIN

FIG. 2. SAME CULTURE FROM THE SURFACE OF AN AGAR SLANT; TWENTY-FOUR HOURS OLD; × 1500; CARBOL THIONIN

FIG. 3. A NEARLY MATURE ZYGOSPORE FORMED AT THE JUNCTION OF TWO CELLS; FROM SURFACE OF A TWENTY-FOUR-HOUR AGAR SLANT; × 1200; CARBOL THIONIN

the final stages in the development of the zygospore. As is characteristic for the process of isoganic conjugation it is located at the junction of two cells, the central chromatin of which is still attached to the developing form. Since the serial development of the process with this strain is shown to much greater advantage in the first paper of this series its repetition here is unnecessary.

The fact that these zygospores when inoculated into broth could, under the warm stage, be observed to reproduce as enor-

mous coccoids seems to leave no doubt that the coarse fungoid pleomorphic stage of the original culture had been dissociated from the stage showing "normal" morphology. Moreover the fact that this stage, although reproducing as a coccoid in broth, was shown to have a filamentous or rod phase on agar, which gave rise directly to the zygospores, and that these again germinated as coccoids and cocci in the broth, not only completes the cycle but identifies *this fungoid variant* with the zygospore producing phase of the original culture. Occasion is taken again to point out that this mechanism of dissociation is no different from that previously pointed out by us (see list of references, Mellon, 1925) and of which other examples will be given as the series progresses.

When kept in rapid transplant the strain was stable and the morphological picture constant, but when standing for longer periods than a few weeks the tendency was definitely for a reversion to the original morphology where the smaller bacillary form dominates. Single celled cultures ruled out the possibility of these smaller forms being present in the large fungoid culture as dissociated from the original Nx strain.

That this fungoid stage characterized by the zygospore process and by the absence of normal sized *B. coli* is associated with functional aberrancy is clearly indicated by its failure to ferment lactose with either acid or gas. This is in marked contrast to the original culture which ferments the sugar violently with acid and gas within twelve hours. The agglutination reactions of the two strains showed them to be serologically identical. Some months later one of the older cultures of this strain dissociated a slender fairly long type of organism which fermented lactose broth to a pH of 6.1 after six days but produced no gas. Its serology still remained unchanged.

This delayed lactose fermenting variant which represents an incomplete reversion of the wild fungoid type toward the normal actively fermenting type, *is in reality the classical B. coli-mutabile whose obscurity genetically now seems to be clear.* When plated on Endo it developed after twenty-four hours medium sized "white" colonies in which after forty-eight to ninety-six hours at 37°

secondary or daughter colonies appeared, some of which were "red" and others "white."

Now, at this stage we found that if the "red" secondary colonies were plated, a mixture of pure "red" and pure "white" colonies were formed after twenty-four hours, and from then on the "red" ones bred true, as did the "white" ones except as the latter were permitted to dissociate the "red" secondary colonies by prolonged incubation. Irrespective of whether the secondary colonies were "white" or "red" they yielded acid on transfer, while transfer from the periphery of the colony where no daughter colonies were present invariably yielded pure "white" colonies. This strain is representative of Massini's *B. coli-mutabile, and was derived genetically from the non-lactose fermenting fungoid stage of B. coli.* The latter strain was in its turn dissociated from a pure line typical *B. coli* vigorously fermenting lactose with both acid and gas. The *B. coli-mutabile* strain did not regain its capacity to ferment lactose *with gas.*

Further experiments served to explain why all the bacilli in a given colony could not develop to a point where lactose was produced. It appeared that the reaction itself, or some metabolic products associated with it, inhibited this evolution on the part of the remainder of the bacilli, inasmuch as adjacent pure "white" colonies which were subject to the diffusion of such products from the "red" colonies *would not form secondary colonies even after two weeks.* That their capacity to do so was merely inhibited was clearly revealed by their transfer to fresh Endo where numerous secondary "red" colonies developed after the usual time interval. It would seem clear that Massini's *B. coli-mutabile* involves primarily an atavistic reversion of the normal *B. coli* to its wild fungoid or saprophytic state that is not capable of secreting the enzyme lactase. This non-lactose fermenting form may again develop this function, *but only when the organism can pass through that phase of its cycle where lactase is secreted.* Moreover, that phase is represented by the secondary colonies whose significance with this strain differs in no fundamental way from that previously pointed out by us for a diphtheroid bacillus (1922).

These changes in behaviour toward lactose that give character to *B. coli-mutabile* are in a sense the counterpart of the chromatic reorganizations that are *partly* traceable in the changed morphology of zygospore formation. I wish to emphasize that this interpretation of cyclic change is by no means an attempt to correlate functional change with *any* indiscriminate morphologic alteration. In point of fact the Massini strain that we have dissociated from the non-lactose fermenting fungoid strain differs in no essential morphologic particular from the original Nx strain; *yet this fact standing alone should not be permitted to obscure the functional importance of the sexual cycle that conditions these changes, merely because zygospore formation has a definite morphologic counterpart. This strain of B. coli-mutabile is essentially a transition stage in the cycle between the wild non-lactose fermenting coli and the normal fermenting form. Its "white" primary colonies actually produce alkali and from them spring "red" secondary colonies which produce acid.*

The relatively unstable character of this fungoid phase makes it clear that its biologic significance, associated as it was with latency of character, would have been quite overlooked had the morphology not been followed closely. It is of further interest that the reversion of this anomalous form to the more normal form developed a strain of somewhat delayed lactose fermentation with which the formation of gas has to date not been associated. In connection with the repeatedly observed fact that these atypical non-lactose, or delayed lactose-fermenting, strains are frequently isolated from the diseased intestine or the blood, their existence now has adequate explanation.

In point of fact the Nx strain when first isolated from the urine was of such character, and it was only after several transplants that it developed its vigorous fermentative ability. The fact that this stage of the organism was reproduced from a single celled culture of a rapidly fermenting organism by passing through what we regard as the sexual phase is of considerable interest in the origin of these variants.

It indicates also *that only certain cells of a culture* may ferment a sugar and then only when they have reached a

stage of development that permits liberation of the enzyme. Thus there appears to be a physiological division of fermentative activity among the cells of a culture which is roughly comparable to the segregation of group and specific agglutinogens which Andrewes (1922) has shown in pure cultures of various members of the Salmonella group.

These experiments testify further to the exact botanical nature of the adaptive mechanism of bacteria under different conditions of environment, another aspect of which has been treated in a previous paper (Mellon, 1922). The present study reveals a similarity with certain features of a work by Prell (1917) who has dissociated the bacillary and the coccus phases of *B. coli* which he styles respectively the rhabdocytic and coccocytic phases. He regards these phases as separate generations in a developmental cycle and believes them to be connected with the differentiation of closely related types. His only objection to calling them mutants is the fact that sexuality has never been demonstrated for bacteria. This has forced him to an unnatural classification of the many variants that he has observed, which from the systematic point of view is really confusing. I believe, however, that his opinion that these phasic changes indicate the essentially fungus nature of bacteria is close to the truth.

CONCLUSIONS

1. It has been shown that the origin, genetically speaking, of Neisser and Massini's *B. coli-mutabile* is definitely associated with what is regarded as a sexual reorganization, or its equivalent, which occurs with this strain.

2. Delay or loss of capacity for the fermentation of a sugar such as occurs with *B. coli-mutabile* appears to involve the *truly pleomorphic cycle* that these organisms undergo, which does not of necessity imply that the change in characters is *permanently* identified with a fixed morphologic type.

3. *B. coli-mutabile* has been reproduced experimentally. It springs from the wild non-lactose fermenting *B. coli* which in turn was derived from a "normal" strain; it is a transition developmental stage (variant) between these two.

4. The simultaneous or sequential production of acid and alkali by a pure line culture appears to be a function of its capacity to develop *phases* of growth. Accordingly the loss of *B. coli's* ability to ferment lactose means that the developmental phase bearing this character (the secondary colonies) is absent or undeveloped.

5. These observations are best explained on the ground that bacteria are fungi whose life cycle is not completely known, or at least is not always completely manifested under the conditions of observation, i.e. they are fungi imperfecti. The cultural and serological heterogeneity which bacteriologists are slowly apprehending finds a most logical explanation on this basis.

REFERENCES

ADAMI, J. G., ABBOTT, M. E., AND NICHOLSON, F. J. 1899 On the diplococcoid form of the colon bacillus. Jour. Exper. Med., **4**, 349–372.

ANDREWES, F. W. 1922 Studies in group agglutination. I. The Salmonella group and its antigenic structure. Jour. Path. and Bact., **25**, 505–521.

ARKWRIGHT, J. A. 1921 Variation in bacteria in relation to agglutination both by salts and specific sera. Jour. Path. and Bact., **24**, 36–60.

BERGSTRAND, HILDING 1923 On the variation of bacterium coli. Jour. Bact., **8**, 173–192.

BERGSTRAND, HILDING 1924 On variation of bacteria. Acta Pathol. et Microbiol. Scandinavica (Copenhagen), **1**, 105–113.

BREINL, F. 1922 Variationserscheinungen in der Dysenterie-gruppe. Ztschr. f. Immunitätsforsch. (Jena), Orig., **35**, 176–190.

D'HERELLE, F. 1922 The bacteriophage: Its Rôle in Immunity. Baltimore, The Williams and Wilkins Co., 287 pp.

EISENBERG, PHILIP 1918 Untersuchungen über der Variabilität der Bakterien. VI. Mitteilung. Centralbl. f. Bakt., Abt. I, Orig., **80**, 385–413.

KOWALENKO, A. 1910 Studien über sogenannte Mutationserscheinungen bei Bakterien, etc. Ztschr. f. Hyg., **66**, 2, 277–290.

MASSINI, R. 1907 Über einen in biologischer Beziehung interessanten Kolistamm (Bact. coli mutabile). Arch. f. Hyg., **61**, 3, 250–292.

NEISSER, M. 1906 Ein Fall von Mutation nach de Vries bei Bakterien. Centralbl. f. Bakt., I. Abt., Ref. **36**, Beiheft, p. 98.

MELLON, R. R., ACREE, S. F., AVERY, PAULINE AND SLAGLE, E. A. 1921 The ionization constants of glycerophosphoric acid and their use as buffers, especially in culture mediums. Jour. Inf. Dis., **29**, 1–6.

MELLON, R. R. 1922 Spontaneous agglutination of bacteria in relation to variability and to the action of equilibrated solutions of electrolytes. Jour. Med. Res., **43**, 3, 345–367.

MELLON, R. R. 1925 Studies in microbic heredity. I. Observations on a primitive form of sexuality (zygospore formation) in the colon-typhoid group. Jour. Bact., **10**, 5, 481–501.

PENFOLD, W. J. 1911 Further experiments on variability in the gas-forming power of intestinal bacteria. Jour. Hyg., **11**, 487.

PRELL, H. 1917 Die Vielgestaltigkeit des Bacterium coli. Centralbl. f. Bakt., I. Abt., Orig., **79**, 6, 324–339.

RODET, A. 1894 La variabilité dans les microbes. Paris (from a quoted reference that did not give the journal).